EAGLE BIOSCIENCES

Free Testosterone LIA Assay Kit

Catalog Number:
TSF31-L01 (1 x 96 wells)
For Research Use Only. Not for use in diagnostic procedures.
v. 1.0

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INTENDED USE

The Eagle Biosciences Free Testosterone LIA Assay Kit is for the direct quantitative determination of free testosterone in human serum by a chemiluminescence immunoassay (LIA). The Eagle Biosciences Free Testosterone 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 ovaries 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 the circulating testosterone is derived from this origin. Testosterone measurements are used mainly for clinical evaluation of hypogonadism in males and hyperandrogenic states in females. Testosterone circulates in the blood bound to three proteins: sex hormone binding globulin (60–80%), albumin and cortisol binding globulin. Only about 1–2% of the total circulating testosterone remains unbound or free. Even though it is still under investigation, most researchers accept the free testosterone determination as a measure of the biologically active fraction. Free testosterone determinations are recommended to overcome the influences caused by variations in transport proteins on the total testosterone concentration.

PRINCIPLE OF THE ASSAY

The principle of the following chemiluminescence immunoassay (LIA) test follows the typical competitive binding scenario. Competition occurs between an unlabelled antigen (present in standards, control and patient samples) and an enzyme-labelled antigen (conjugate) for a limited number of antibody binding sites on the microplate. The washing and decanting procedures remove unbound materials. After the 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 free testosterone in the sample. A set of calibrators are used to plot a standard curve from which the amount of free testosterone in patient samples and controls can be directly read. The labelled testosterone (conjugate) employed in this assay system has shown no binding properties towards SHBG and human serum albumin. A highly specific rabbit anti-testosterone polyclonal antibody at a low binding capacity (Keq x concentration) is used to keep minimum disturbances of the testosterone-protein equilibrium. The other components in the test system are also optimized in order to not alter the original free testosterone concentration.

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 free testosterone in human serum. The kit is not calibrated for the determination of free testosterone in saliva, plasma or other specimens of human or animal origin.
2. Do not use grossly hemolyzed, grossly lipemic, icteric or improperly stored serum.
3. Any samples or control sera containing azide or thimerosal are not compatible with this kit, as they may lead to false results.
4. Samples reading higher than the highest calibrator should be reported as such and should not be diluted. Dilution will alter the existing equilibrium and may lead to false results.
5. This kit is intended for research use only and should not be used in diagnostic procedures.

SAFETY CAUTIONS AND WARNINGS
POTENTIAL BIOHAZARDOUS MATERIAL

Human serum 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 0.2 mL of serum is required per duplicate determination. Collect 4–5 mL of blood into an appropriately labelled tube and allow it to clot. Centrifuge and carefully remove the serum layer. Store 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

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

REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED

1. Precision pipettes to dispense 25, 100, 150 and 300 µL
2. Disposable pipette tips
3. Distilled or deionized water
4. A 37°C incubator
5. Plastic wrap or microplate cover
6. Microplate luminometer

REAGENTS PROVIDED AND PREPARATION

1. Rabbit Anti-Free 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. Free Testosterone Horseradish Peroxidase (HRP) Conjugate Concentrate — Requires Preparation X50

   Contents: Free testosterone-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 conjugate concentrate 1:50 in assay buffer before use (example: 20 µL of conjugate concentrate in 1 mL of assay buffer). If the whole plate is to be used dilute 240 µL of conjugate concentrate in 12 mL of assay buffer. Discard any that is left over.

3. Free Testosterone Calibrators — Ready To Use

   Contents: Six vials containing testosterone in a human serum-based buffer with a non-mercury preservative. Prepared by spiking serum with a precise quantity of testosterone equivalent to approximately 0, 0.25, 1, 5.5, 25 and 125 pg/mL of free testosterone.
   * Listed below are approximate concentrations, please refer to bottle labels for exact concentrations.

Calibrator	Concentration	Volume/Vial
Calibrator A	0 pg/mL	0.5 mL
Calibrator B	0.25 pg/mL	0.5 mL
Calibrator C	1 pg/mL	0.5 mL
Calibrator D	5.5 pg/mL	0.5 mL
Calibrator E	25 pg/mL	0.5 mL
Calibrator F	125 pg/mL	0.5 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.

4. Controls — Ready To Use
   - Contents: Two vials containing testosterone in a human serum- based buffer with a non-mercury preservative. Prepared by spiking serum with a precise quantity of testosterone. Refer to vials labels for expected value and acceptable range.
   - Volume: 0.5 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.

5. 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.

6. Assay Buffer
   - Contents: One bottle containing protein-based buffer with a non-mercury preservative.
   - Volume: 15 mL/bottle
   - Storage: Refrigerate at 2–8°C
   - Stability: 12 months or as indicated on label.

7. Rinse Solution – Ready To Use
   - Contents: Two bottles containing buffer with a non-mercury preservative.
   - Volume: 2x 50 mL/bottle
   - Storage: Refrigerate at 2–8°C
   - Stability: 12 months or as indicated on label.

8. 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.

9. 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.

10. LIA Substrate Reagent C – Requires Preparation

Contents:	One bottle containing buffer with a non-mercury preservative.
Volume:	16 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 0.45 mL of LIA substrate reagent A with 0.9 mL of LIA substrate reagent B and 9 mL of LIA substrate reagent C. It is suggested to wait at least 30 minutes prior to use after mixing of the reagent A, B and C. The working substrate solution is stable for up to 8 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.

1. Prepare working solutions of the conjugate, 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 25 µL of each calibrator, control and specimen sample into correspondingly labelled wells in duplicate.
4. Pipette 100 µL of the conjugate working solution into each well (We recommend using a multichannel pipette).
5. Cover the plate and incubate for 1 hour in a 37°C incubator.
6. Wash the wells 3 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. Rinse the wells 3 times with 300 µL of rinse solution per well and tap the plate against absorbent paper to ensure it is dry.
8. Pipette 130 µL of LIA working substrate solution into each well. (We recommend using a multichannel pipette.)
9. Measure the RLU/second in each well on a microplate luminometer between 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. Samples reading higher than the highest calibrator should be reported as such and should not be diluted. Dilution will alter the existing equilibrium and may lead to false results.

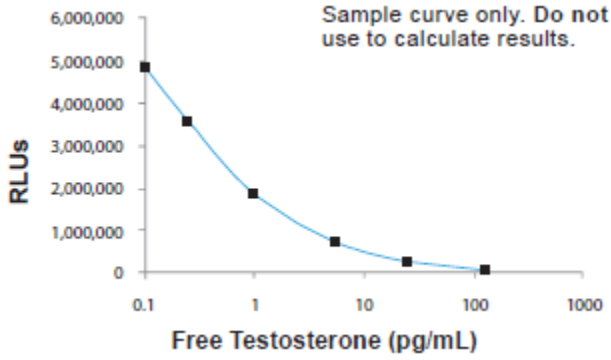
TYPICAL TABULATED DATA**

Sample data only. Do not use to calculate results.

Calibrator	RLU 1	RLU 2	Mean RLU	RLU/RLU$_{MAX}$
A, 0 pg/mL	4881890	4806080	4843985	100%
B, 0.25 pg/mL	3736040	3374440	3555240	73%
C, 1 pg/mL	1873680	1814940	1844310	38%
D, 5.5 pg/mL	682900	737040	709970	15%
E, 25 pg/mL	257710	246700	252205	5.2%
F, 125 pg/mL	35730	34860	35295	0.7%

** 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 free testosterone LIA kit is **0.17 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	1.08	0.11	9.8
2	13.59	0.81	5.9
3	65.86	4.48	6.8

INTER-ASSAY PRECISION

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

Sample	Mean	SD	CV %
1	1.23	0.15	12.1
2	14.53	1.21	8.3
3	63.47	5.59	8.8

COMPARATIVE STUDIES

The free testosterone LIA kit (y) was compared with the free testosterone ELISA kit (x). The comparison of 50 serum samples yielded the following linear regression results:

$y = 0.9256 X + 0.338,\ r2 = 0.99$

EFFECT OF SEX HORMONE BINDING GLOBULIN (SHBG)

The purpose of this study was to investigate a possible interference caused by the binding of SHBG to the free testosterone-horse radish peroxidase conjugate. A charcoal-stripped human serum pool was spiked precisely with SHBG at concentrations ranging from 6–200 µg/mL and was assayed with the free testosterone LIA Kit. Results tabulated below (in pg/mL):

SHBG Added	RLU ($x10^6$)	Percent B/B_0 (%)
0 µg/mL	1.55	100.0
6.25 µg/mL	1.54	99.7
12.5 µg/mL	1.51	97.2
50 µg/mL	1.42	91.6
200 µg/mL	1.39	89.7

The results showed bound values between 90–100% of B/B_0 (B_0 = unspiked serum) even at higher than normal (0.5–5 µg/mL) SHBG levels. In conclusion, the results showed that there was no significant influence by SHBG in the free testosterone LIA kit.

EFFECT OF HUMAN SERUM ALBUMIN (HSA)

The purpose of this study was to investigate a possible interference of human serum albumin (HSA) on the assay procedure. HAS was added to three patient samples at concentrations of 1.25, 2.5 and 5.0 g/dL. All samples were assayed with the free testosterone LIA Kit and yielded the following results (in pg/mL):

Sample	Added HAS g/dL			
	0	1.25	2.5	5.0
1	0.52	0.34	0.54	0.53
2	15.8	14.2	12.5	10.9
3	26.2	23.0	21.0	18.6

The results demonstrate no significant influence of added HSA on the three patient serum samples.

EXPECTED NORMAL VALUES

As for all clinical assays each laboratory should collect data and establish their own range of expected normal values. The results of an expected range study with apparently normal healthy subjects yielded the following results (all values are reported in pg/mL):

Group	N	Median	Central 95% Range	Absolute Range
Males	71	12.3	4.25-30.37	3.84-34.17
Females	60	1.03	0.04-4.18	0.01-7.01

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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.

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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.