



**Diagnostics Biochem Canada Inc.**

Manufacturer of In Vitro Diagnostic Test Kits Since 1973

## Chemiluminescence Immunoassay (LIA)

# Free Testosterone

Rapid, Sensitive, Direct Microtiter Strip Immunoassay

Cat. No.: CAN-FTE-6080

Version: 1.1

Effective: Oct 25,2005

### INTENDED USE

For the direct quantitative determination of Free Testosterone in human serum by chemiluminescence immunoassay. For *in vitro* use only.

### PRINCIPLE OF THE TEST

The principle of the following chemiluminescence immunoassay (LIA) test follows the typical competitive binding scenario. Competition occurs between an unlabeled antigen (present in standards, control and patient samples) and an enzyme-labelled antigen (conjugate) for a limited number of antibody binding sites on the microwell plate. 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 labeled 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 (K<sub>eq</sub> 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.

### CLINICAL APPLICATIONS

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.

### PROCEDURAL CAUTIONS AND WARNINGS

- 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.
- Control materials or serum pools 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 kit 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.
- The luminescence substrate solutions (A and B) are sensitive to light and should be stored in the original dark bottle away from direct sunlight.
- When dispensing the substrate, do not use pipettes in which this 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 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.
- Do not use grossly hemolyzed, grossly lipemic, icteric or improperly stored serum.
- Any samples or control sera containing azide or thimerosal are not compatible with this kit, as they may lead to false results.
- Samples reading higher than the highest standard should be reported as such and should not be diluted. Dilution will alter the existing equilibrium and may lead to false results.
- The results obtained with this kit should never be used as the sole basis for 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

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 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

- Precision pipettes to dispense 25, 100, 150 and 300 µl
- Disposable pipette tips
- Distilled or deionized water
- A 37°C incubator
- Plastic wrap or micro-plate cover.
- Microwell plate luminometer

### REAGENTS PROVIDED AND PREPARATION

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

Contents: Free Testosterone-HRP 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 1:100 in assay buffer before use (eg. 20 µl of HRP in 2 ml of assay buffer). If the whole plate is to be used dilute 120 µl of HRP in 12ml 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, 25 and 100 pg/ml of free testosterone.

\*Listed below are approximate concentrations, please refer to vial 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 pg/ml	0.5 ml
Calibrator E	25 pg/ml	0.5 ml
Calibrator F	100 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. Control - Ready To Use.

Contents: One vial 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 vial label for expected value and acceptable range.

Volume: 0.5 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.

#### 5. 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.

#### 6. Assay Buffer - Ready To Use.

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

Volume: 15 ml/vial

Storage: Refrigerate at 2-8°C

Stability: 12 months or as indicated on label.

#### 7. Chemiluminescence Substrate Reagent A - Requires Preparation.

Volume: 1.5 ml/bottle

Storage: Refrigerate at 2-8°C

Stability: as indicated on label.

Preparation: See below.

#### 8. Chemiluminescence Substrate Reagent B - Requires Preparation.

Volume: 1.5 ml/vial

Storage: Refrigerate at 2-8°C

Stability: as indicated on label.

Preparation: See below.

#### 9. Chemiluminescence Substrate Reagent C - Requires Preparation.

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

Volume: 16 ml/vial

Storage: Refrigerate at 2-8°C

Stability: as indicated on label.

Preparation: See below.

### Preparation of Working Substrate Solution:

In a clean plastic container (glass is not suitable) mix 1 part of the substrate reagent A with 1 part of reagent B and 10 parts of 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 1.0 ml of reagent A with 1.0 ml of reagent B and 10 ml of 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**Specimen Pretreatment: *None.***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.
- Remove the required number of microwell strips. Reseal the bag and return any unused strips to the refrigerator.
  - Pipette 25 µl of each calibrator, control and specimen sample into correspondingly labelled wells in duplicate.
  - Pipette 100 µl of the conjugate working solution into each well (We recommend using a multichannel pipette).
  - Cover the plate and incubate for 60 minutes in a 37 °C incubator.
  - 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).
  - Pipette 100 µl of chemiluminescence substrate working solution into each well (We recommend using a multichannel pipette).
  - Incubate for 10-15 minutes at room temperature.
  - Measure the RLU/second in each well on a microplate luminometer within 10-30 minutes after addition of the substrate.

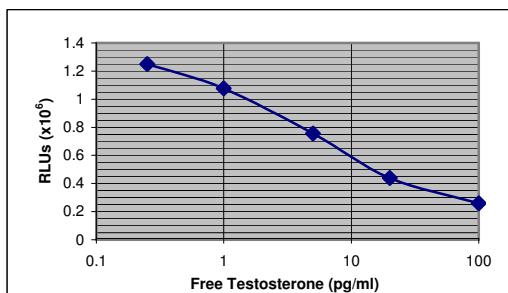
**CALCULATIONS**

- Calculate the mean RLU of each calibrator duplicate.
- 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.
- Calculate the mean RLU of each unknown duplicate.
- Read the values of the unknowns directly off the calibrator curve.
- 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\*\***

Calibrator	RLU 1 x 10 <sup>3</sup>	RLU 2 x 10 <sup>3</sup>	Mean RLU x 10 <sup>3</sup>	RLU/RLU <sub>MAX</sub> (%)
A, 0 pg/ml	1500	1434	1467	100
B, 0.25 pg/ml	1240	1260	1250	87
C, 1 pg/ml	1044	1110	1077	61
D, 5 pg/ml	716	796	756	36
E, 20 pg/ml	452	428	440	15
F, 100 pg/ml	250	272	261	4.3

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

**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 RLU of Calibrator A (based on 10 replicate analyses) minus 2 SD. Therefore, the sensitivity of the dbc Direct Free Testosterone LIA kit is **0.17 pg/ml**.

**SPECIFICITY (CROSS REACTIVITY)**

The following compounds were tested for cross-reactivity with the dbc Direct Free Testosterone LIA kit with testosterone cross-reacting at 100%.

Steroid	%Cross Reactivity
Testosterone	100
5α-DHT	5.2
Androstenedione	1.4
Androstenediol	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 dbc Direct Free Testosterone LIA kit (y) was compared with dbc Direct Free Testosterone ELISA kit (x). The comparison of 50 serum samples yielded the following linear regression results:

$$y=0.9256 X + 0.338$$

$$r^2=0.99$$

**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 dbc Direct Free Testosterone LIA Kit. Results tabulated below (in pg/ml):

SHBG Added	RLU (x10 <sup>5</sup> )	Percent B/B <sub>0</sub> (%)
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<sub>0</sub> (B<sub>0</sub>=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 dbc Direct Free Testosterone Direct 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. HSA was added to three patient samples at concentrations of 1.25, 2.5 and 5.0 g/dl. All samples were assayed with the Direct dbc Free Testosterone LIA Kit and yielded the following results (in pg/ml):

Sample	Added HSA 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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**OTHER RELATED DBC KITS**

Also available from stock are the following dbc kits:

- dbc Direct Free Testosterone ELISA Kit, Cat. No.:CAN-FTE-260
- dbc Direct Total Testosterone ELISA Kit, Cat. No.:CAN-TE-250
- dbc Testosterone Saliva ELISA Kit, Cat. No.:CAN-TE-300

Please contact us if you require any further information.

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