DRG® Testosterone free (CLA-4660)

REVISED 31 MAR. 2011 RM (VERS. 4.1) **RUO IN THE USA**

Please use only the valid version of the package insert provided with the kit.

This kit is intended for Research Use Only.

Not for use in diagnostic procedures.

1 INTENDED USE

For the determination of free testosterone in human serum by a chemiluminescence immunoassay.

2 PRINCIPLE OF THE TEST

The principle of the following chemiluminescence immunoassay (CLIA) test follows the typical competitive binding scenario. Competition occurs between an unlabeled antigen (present in standards, control and 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 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 (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.

3 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 control 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 control do not reflect established ranges.
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.

4 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 SAFETY CAUTIONS AND WARNINGS

POTENTIAL BIOHAZARDOUS MATERIAL

Human serum that may be used in the preparation of the standards and control have been tested and found to be non-reactive for Hepatitis B surface antigen and have 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 human specimen.

CHEMICAL HAZARDS

Avoid direct contact with reagents. In case of contact, wash with plenty of water.

6 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.

7 SPECIMEN PRETREATMENT

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

8 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 micro-plate cover.
6. Microwell plate luminometer

9 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 - 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 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	5.5 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. Control - 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 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 of wash buffer working solution:
Dilute wash buffer concentrate 1:10 in distilled or deionized water before use. If the whole plate is to be used dilute 50 mL of wash buffer concentrate in 450 mL of water.

6. Assay Buffer - Ready To Use.
Contents: One bottle containing a 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. CLIA Substrate Reagent A - Requires Preparation.
Contents: One vial containing luminol and enhancers.
Volume: 0.8 mL/vial
Storage: Refrigerate at 2-8°C
Stability: as indicated on label.
Preparation: See preparation of LIA working substrate solution.

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

10. CLIA 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: as indicated on label.
Preparation: See preparation of LIA working substrate solution.

Preparation of CLIA Working Substrate Solution

In a clean container mix **1 part** of LIA substrate **reagent A** with **2 part** 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.

10 ASSAY PROCEDURE

Important Notes:

1. All reagents must reach room temperature before use.
2. 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 microwell 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 10-20 minutes after addition of the substrate.

11 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.

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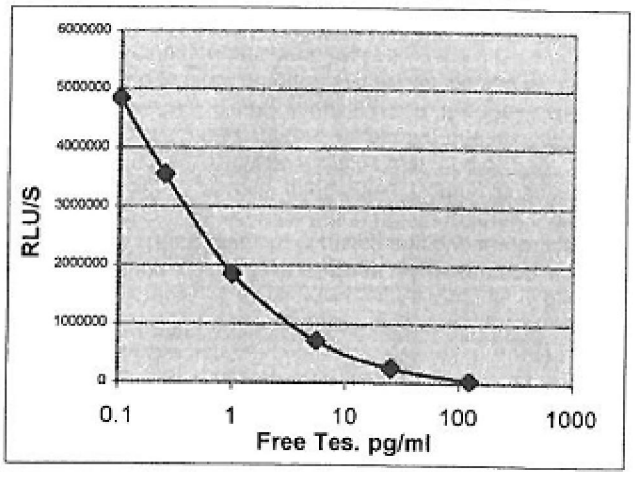
TYPICAL TABULATED DATA**

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

Sample curve only. **Do not** use to calculate results.



11.1 COMPARATIVE STUDIES

The free testosterone LIA kit (CLA-4660) (y) was compared with the free testosterone ELISA kit (x). The comparison of 50 serum samples yielded the following linear regression results:

$y = 0.9256 X + 0.338$, $r^2 = 0.99$

11.2 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 DRG free testosterone LIA Kit. Results tabulated below (in pg/mL):

SHBG Added	RLU ($x10^6$)	Percent B/B_o (%)
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/Bo (Bo=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 DRG free testosterone LIA kit.

11.3 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 samples at concentrations of 1.25, 2.5 and 5.0 g/dL. All samples were assayed with the DRG 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 serum samples.

12 REFERENCES

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