Monobind Inc.
Lake Forest, CA 92630, USA

AccuLite
CLIA Microwells

Free Testosterone Test System

Product Code: 5375-300

1.0 INTRODUCTION

Intended Use: The Quantitative Determination of Free Testosterone Concentration in Human Serum or Plasma by a Microplate Enzyme Immunoassay, Chemiluminescence.

2.0 SUMMARY AND EXPLANATION OF THE TEST

Testosterone, (17β-Hydroxy-4-androstene-3-one), a C_{19} steroid, is the most potent naturally secreted androgen[1]. In normal post pubertal males, testosterone is secreted primarily by the testes with only a small amount derived from peripheral conversion of 4-Androstene-3, 17-dione (ASD)[2]. In adult women, it has been estimated that over 50% of serum testosterone is derived from peripheral conversion of ASD secreted by the adrenal and ovary, with the remainder from direct secretion of testosterone by these glands.

In the male, testosterone is mainly synthesized in the interstitial Leydig cells and the testis, and is regulated by the interstitial cell stimulating hormone (ICSH), or luteinizing hormone (LH) of the anterior pituitary (the female equivalent of ICSH)[3]. Testosterone is responsible for the development of secondary sex characteristics, such as the accessory sex organs, the prostate, seminal vesicles and the growth of facial, pubic and auxiliary hair. Testosterone measurements have been very helpful in evaluating hypogonadal states. Increased testosterone levels in males can be found in complete androgen resistance (testicular feminization). Common causes of decreased testosterone levels in males include: hypogonadism, orchidectomy, estrogen therapy, Klinefelter's syndrome, hypopituitarism, and hepatic cirrhosis[2-4].

In the female, testosterone levels are normally found to be much lower than those encountered in the healthy male. Testosterone in the female comes from three sources. It is secreted in small quantities by both the adrenal glands and the ovaries, and in healthy women 50–60% of the daily testosterone production arises from peripheral metabolism of prohormone, chiefly androstenedione. Common causes of increased serum testosterone levels in females include polycystic ovaries (Stein-Leventhal syndrome), ovarian tumors, adrenal tumors and adrenal hyperplasia. Virilization in women is associated with the administration of androgens and endogenous overproduction of testosterone. There appears to be a correlation between serum testosterone levels and the degree of virilization in women, although approximately 25% of women with varying degrees of virilism have serum testosterone levels that fall within the female reference range.

The majority of testosterone is bound to transport proteins; weakly bound to albumin and cortisol binding protein (25-65% females - 45-85% males) and tightly bound to sex hormone-binding globulin (SHBG) (females 35-75% - males 14-50%)[8]. A small fraction exist as unbound or free testosterone, however this form is biologically active. Therefore, the free hormone concentration is a better indicator of biological activity than total testosterone.

3.0 PRINCIPLE

Competitive Enzyme Immunoassay (TYPE 5):
The essential reagents required for a solid phase enzyme immunoassay include immobilized antibody, enzyme-antigen conjugate and native antigen.

Upon mixing immobilized antibody, enzyme-antigen conjugate and a serum containing the native antigen, a competition reaction results between the native antigen and the enzyme-antigen conjugate for a limited number of insolubulized binding sites. The interaction is illustrated by the equation in the following below.

$$^{Enz}Ag + Ag + Ab_{C.W.} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} AgAb_{C.W.} + {}^{Enz}AgAb_{C.W.}$$

$Ab_{C.W}$ = Monospecific Immobilized Antibody (Constant Quantity)
Ag = Native Antigen (Variable Quantity)
^{Enz}Ag = Enzyme-antigen Conjugate (Constant Quantity)
$AgAb_{C.W.}$ = Antigen-Antibody Complex
$^{Enz}AgAb_{C.W.}$ = Enzyme-antigen Conjugate -Antibody Complex
k_a = Rate Constant of Association
k_{-a} = Rate Constant of Disassociation
$K = k_a / k_{-a}$ = Equilibrium Constant

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is inversely proportional to the native antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

4.0 REAGENTS

Materials Provided:

A. Free Testosterone Calibrators* – 1ml/vial – Icons A-G
Seven (7) vials of serum reference for Free Testosterone at **approximate*** concentrations of 0 (**A**), 0.2 (**B**), 1.0 (**C**), 2.5 (**D**), 7.5 (**E**), 20 (**F**) and 60 (**G**) in pg/ml. Store at 2-8°C. A preservative has been added. The calibrators can be expressed in molar concentrations (pM/L) by multiplying by 3.47.For example: 1pg/ml x 3.47 = 3.47 pM/L
Exact levels are given on the labels on a lot specific basis.

B. Free Testosterone Controls – 1ml/vial – Icons L, M, N
Three (3) vials of serum reference for Free Testosterone at low, middle, and high established concentrations (range values listed on labels). A preservative has been added. Store at 2-8°C.

C. Free Testosterone Tracer Reagent – 13 ml/vial – Icon
One (1) vial of Testosterone (Analog)-horseradish peroxides (HRP) conjugate in a protein stabilizing matrix with dye. Store at 2-8°C.

D. Free Testosterone Coated Light Reaction Wells – 96 wells – Icon
One 96-well microplate coated with testosterone antibody and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

E. Wash Solution Concentrate – 20ml/vial – Icon
One (1) vial contains a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

F. Signal A – 7ml/vial – Icon S[A]
One (1) vial contains luminol in a buffer. Store at 2-8°C.

G. Signal B – 7ml/vial – Icon S[B]
One (1) vial contains hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

H. Product Instructions

Note 1: Do not use reagents beyond the kit expiration date.
Note 2: Avoid extended exposure to heat and light. **Opened reagents are stable for sixty (60) days when stored at 2-8°C. Kit and component stability are identified on the test.**
Note 3: Above reagents are for a single 96-well microplate.

4.1 Required But Not Provided:
1. Pipette capable of delivering 20µl, 50µl, and 100µl volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100ml and 0.300ml volumes with a precision of better than 1.5%.
3. Adjustable volume (200-1000µl) dispenser(s) for conjugate.
4. Microplate washer or a squeeze bottle (optional).
5. Microplate Luminometer.
6. Absorbent Paper for blotting the microplate wells.
7. Plastic wrap or microplate cover for incubation steps.
8. Vacuum aspirator (optional) for wash steps.
9. Timer.
10. Quality control materials.

5.0 PRECAUTIONS

For In Vitro Diagnostic Use
Not for Internal or External Use in Humans or Animals

All products that contain human serum have been found to be non-reactive for Hepatitis B Surface Antigen, HIV 1&2 and HCV Antibodies by FDA approved tests. Since no known test can offer complete assurance that infectious agents are absent, all human serum products should be handled as potentially hazardous and capable of transmitting disease. Good laboratory procedures for handling blood products can be found in the Center for Disease Control / National Institute of Health, "Biosafety in Microbiological and Biomedical Laboratories," 2nd Edition, 1988, HHS Publication No. (CDC) 88-8395.

Safe disposal of kit components must be according to local regulatory and statutory requirement.

6.0 SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood, serum or plasma in type and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube or (for plasma) in evacuated tube(s) containing heparin. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.040ml of the specimen is required.

7.0 QUALITY CONTROL

Each laboratory should assay controls at levels in the low, normal and high range for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. The individual laboratory should set acceptable assay performance limits. In addition, maximum absorbance should be consistent with past experience. Significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

8.0 REAGENT PREPARATION

1. **Wash Buffer**
Dilute contents of wash solution to 1000ml with distilled or deionized water in a suitable storage container. Diluted buffer can be stored at 2-30°C for up to 60 days.
2. **Working Signal Reagent Solution -** Store at 2 - 8°C.
Determine the amount of reagent needed and prepare by mixing equal portions of Signal Reagent A and Signal Reagent B in a clean container. For example, add 1 ml of A and 1ml of B per two (2) eight well strips (A slight excess of solution is made). **Discard the unused portion if not used within 36 hours after mixing.** If complete utilization of the reagents is anticipated, within the above time constraint, pour the contents of Signal Reagent B into Signal Reagent A and label accordingly.

Note: Do not use reagents that are contaminated or have bacteria growth.

9.0 TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20 - 27°C).
*****Test procedure should be performed by a skilled individual or trained professional*****

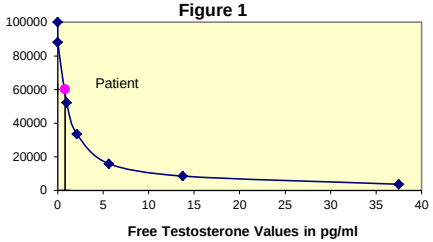
1. Format the microplates' wells for each serum reference, control and patient specimen to be assayed in duplicate. **Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.**
2. Pipette 0.020 ml (20µL) of the appropriate serum reference, control or specimen into the assigned well.
3. Add 0.100 ml (100µl) of the Free Testosterone Tracer Reagent to all wells.
6. Swirl the microplate gently for 20-30 seconds to mix.
7. Cover and incubate for 45 minutes at room temperature.
8. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.
9. Add 350µl of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat four (4) additional times for a total of five (5) washes. **An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat four (4) additional times.**
10. Add 0.100 ml (100µl) of working signal reagent solution to all wells (see Reagent Preparation Section). **Always add reagents in the same order to minimize reaction time differences between wells.**
11. Incubate at room temperature for five (5) minutes in the dark.
12. Read the relative light units in each well with a Chemiluminescence microplate reader for 0.5-1.0 seconds. **The results should be read within 30 minutes after adding the working Signal Reagent.**

10.0 CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of free testosterone in unknown specimens.
1. Record the RLUs obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the RLUs for each duplicate serum reference versus the corresponding free testosterone concentration in pg/ml on linear graph paper.
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of free testosterone for an unknown, locate the average RLUs for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in pg/ml) from the horizontal axis of the graph (the duplicates of the unknown In may be averaged as indicated). In the following example, the average RLUs (60206) of the unknown intersects the calibration curve at (0.82) free testosterone concentration.

* The data presented in Example 1 and Figure 1 is for illustration only and **should not** be used in lieu of a dose response curve prepared with each assay. In addition, the RLU's of the calibrators have been normalized to 100,000 RLU's for the A calibrator (greatest light output). This conversion minimizes differences caused by efficiency of the various instruments that can be used to measure light output.

Figure 1



U

EXAMPLE 1

Sample I.D.	Well Number	RLU (A)	Mean RLU (B)	Value (pg/ml)
Cal A	A1	100406	100000	0.0
	B1	99594		
Cal B	C1	79322	81410	0.2
	D1	83498		
Cal C	E1	40915	41405	1.0
	F1	41895		
Cal D	G1	19838	19256	2.5
	H1	18673		
Cal E	A2	7790	7480	7.5
	B2	7169		
Cal F	C2	2110	2083	20
	D2	2056		
Cal G	E2	439	443	60
	F2	447		
Ctrl L	G2	40973	39204	1.339
	H2	37436		
Ctrl M	A3	11674	11508	5.284
	B3	11341		
Ctrl N	C3	452	523	47.107
	D3	594		
Patient	C4	8387	8305	2.870
	D4	8222		

11.0 Q.C. PARAMETERS

In order for the assay results to be considered valid the following criteria should be met:

1. The Dose Response Curve should be within established parameters.
2. Four out of six quality control pools should be within the established ranges.

12.0 RISK ANALYSIS

The MSDS and Risk Analysis Form for this product is available upon request from Monobind Inc.

12.1 Assay Performance

1. It is important that the time of reaction in each well is held constant to achieve reproducible results.
2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.
3. Highly lipemic, hemolyzed or grossly contaminated specimen(s) should not be used.
4. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
5. The addition of signal reagent initiates a kinetic reaction, therefore the signal reagent(s) should be added in the same sequence to eliminate any time-deviation during reaction.
6. Patient specimens (diluted) with CA 15-3 concentrations above 400 U/ml may be further diluted (1/10 or higher) with CA15-3 diluted serum diluent and re-assayed. The sample's concentration is obtained by multiplying the result by the dilution factor.
7. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
8. Use components from the same lot. No intermixing of reagents from different batches.
9. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from Monobind's IFU may yield inaccurate results.
10. All applicable national standards, regulations and laws, including, but not limited to, good laboratory procedures, must be strictly followed to ensure compliance and proper device usage.
11. It is important to calibrate all the equipment e.g. Pipettes, Readers, Washers and/or the automated instruments used with this device, and to perform routine preventative maintenance.
12. Risk Analysis- as required by CE Mark IVD Directive 98/79/EC - for this and other devices, made by Monobind, can be requested via email from Monobind@monobind.com.

12.2 Interpretation

1. ***Measurements and interpretation of results must be performed by a skilled individual or trained professional.***
2. Laboratory results alone are only one aspect for determining patient care and should not be the sole basis for therapy, particularly if the results conflict with other determinants.
3. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
4. If test kits are altered, such as by mixing parts of different kits, which could produce false test results, or if results are incorrectly interpreted, Monobind shall have no liability.
5. If computer controlled data reduction is used to interpret the results of the test, it is imperative that the predicted values for the calibrators fall within 10% of the assigned concentrations.

13.0 EXPECTED RANGES OF VALUES

In agreement with established reference intervals for a "normal" adult population, the expected ranges for the Free Testosterone AccuBind® CLIA Test System are detailed in Table 1.

TABLE I

Population	Range (in pg/ml)
Male / 20-39	9.2-34.6
Male / 40-59	6.1-30.3
Male / ≥60	6.1-27.9
Female / 20-39	0.2-6.1
Female / 40-59	0.3-4.4
Female / ≥60	0.5-3.4

It is important to keep in mind that establishment of a range of values, which can be expected to be found by a given method for a population of "normal" persons, is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons, each laboratory should depend upon the range of expected values established by the Manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

14.0 PERFORMANCE CHARACTERISTICS

14.1 Precision

The within and between assay precision of the Free Testosterone AccuLite® CLIA Test System were determined by analyses on three different levels of pool control sera. The number, mean values, standard deviation and coefficient of variation for each of these control sera are presented in Table 2 and Table 3.

TABLE 2
Within Assay Precision (Values in pg/ml)

Sample	N	X	σ	C.V.
Level 1	20	3.83	0.18	4.6%
Level 2	20	28.51	0.93	3.2%
Level 3	20	39.46	0.52	1.3%

TABLE 3
Between Assay Precision (Values in pg/ml)

Sample	N	X	σ	C.V.
Level 1	20	3.54	0.24	6.7%
Level 2	20	22.20	2.38	10.7%
Level 3	20	32.81	1.03	3.1%

*As measured in ten experiments in duplicate over a ten day period.

14.2 Sensitivity

The Free Testosterone AccuLite® CLIA Test System has a sensitivity of 0.0024 pg/well. This is equivalent to a sample containing a concentration of 0.120pg/ml. The sensitivity was ascertained by determining the variability of the 0 ng/ml serum calibrator and using the 2σ (95% certainty) statistic to calculate the minimum dose.

14.3 Cross Reactivity

Cross reactivity was determined by testing those compounds most likely to interfere with the Monobind Free Testosterone CLIA Test System. The specificity of the assay was determined in accordance with CLSI EP07-A2. The results of the cross-reactivity study are as follows.

TABLE 4

Sample	Conc. (ng/ml)	Cross Reactivity: Spiked Serum	Cross Reactivity: Blank Serum
11-Deoxycortisol	1000	0.000%	ND
11-KetoTestosterone	10	0.647%	0.519%
11β-Hydroxytestosterone	100	0.065%	0.054%
17α-ethynyl estradiol	1000	0.000%	ND
17α-Estradiol	1000	0.000%	0.000%
17β-Estradiol	100	0.000%	ND
17-Hydroxypregnenolone	1000	0.000%	ND
17-Hydroxprogesterone	10	0.000%	0.000%
3-EstriolGluc	1000	0.000%	ND
3-EstriolSul	1000	0.000%	ND
3β-Androstanediol	500	0.000%	ND
5α-Dihydrotestosterone	100	0.054%	0.042%
Aldosterone	8000	0.000%	0.000%
Amitriptyl HCl	1000	0.000%	ND
Androsterone	1000	0.000%	ND
Androstenedione	1000	0.004%	0.002%
Clomiphene Citrate	1000	0.000%	ND
Corsticosterone	1000	0.000%	0.000%
Corstisone	1000	0.000%	0.000%
Cortisol	1000	0.000%	0.000%
Cyproterone acetate	1000	0.000%	ND
D-5-Androstene-3β,17β-diol	1000	0.000%	ND
Danazol	1000	0.000%	ND
DHEA	100000	0.000%	0.000%
DHEA-S	1000	0.000%	0.000%
Desogestrel	100	0.000%	ND
Dexamethasone	1000	0.000%	ND
Epistestosterone	1000	0.001%	0.001%
Estriol	1000	0.000%	0.000%
Estrone	1000	0.000%	0.000%
Ethisterone	1000	0.000%	0.000%
Ethynodiol	1000	0.000%	0.000%
Ethynodiol diacetate	50	0.000%	ND
Flunisolide	1000	0.000%	ND
Fluoxymesterone	1000	0.000%	ND
Lynestrol	1000	0.000%	ND
Medoxyprogesterone acetate	1000	0.000%	ND
Methyl Testosterone	100	0.000%	ND
Mestranol	1000	0.000%	ND
Norethindrone	50	0.000%	ND
Norethinodrone acetate	50	0.000%	ND
Norgestimate	1000	0.000%	ND
Norgestrel (Levonorgestrel)	50	0.000%	ND
Norethynodrel	50	0.000%	ND
Oxymetholone	100	0.000%	ND
Prednisolone	1000	0.000%	ND
Prednisone	800	0.000%	0.000%
Progesterone	1000	0.000%	0.000%
Salbutamol	1000	0.000%	ND
Spironolactone	1000	0.000%	0.000%
Stanozolol	1000	0.000%	0.000%
Testosterone Cypionate	12	0.002%	0.000%
Testosterone enanthate	100	0.000%	0.000%
Testosterone SO4	1000	0.004%	0.003%
Testosterone Propionate	1000	0.000%	0.000%
Testo Undecanoate	12	0.011%	0.053%
Triamcinolone	50	0.000%	0.000%

14.4 Interference

Using CLSI-A2 Interference Testing in Clinical Chemistry as a guide, potential interferents were tested utilizing charcoal-stripped human serum spiked with known concentrations of interferent. The following results of % binding values even at higher than normal interferent levels indicate that there is no significant binding on the free testosterone-HRP conjugate.

TABLE 5

Substance	Highest concentration at which no significant interference was observed
Acetaminophen	20 mg/dl
Acetylcysteine	150 mg/dl
Ascorbic Acid	6 mg/dl
Bilirubin Conjugated	15 mg/dl
Bilirubin Unconjugated	20 mg/dl
Biotin	100 ng/ml
Caffeine	6 mg/dl
Cholesterol	503 mg/dl
Creatine	30 mg/dl
Dextran	5000 mg/dl
Digoxin	6.1 ng/ml
Doxycycline	50 mg/L
Erythromycin	6 mg/dl
Gentamicin	1 mg/dl
HAMA	440 ng/ml
Heparin	3 U/ml
Hemoglobin	500 mg/dl
Human Serum Albumin	2.5 g/dl
Ibuprofen	50 mg/dl
Immunoglobulin G	4 g/dl
Levodopa	20 mg/L
Lidocaine	1.2 mg/dl
Lipemia (glycerides)	1000 mg/dl
Methyldopa	20 mg/L
Nicotine	0.1 mg/dl
Phenobarbital	15 mg/dl
Protein: Total	10.5 g/dl
Rheumatoid Factor	1110 IU/ml
Salicylic Acid	60 mg/dl
SHBG	200 µg/ml
Triglycerides	900 mg/dl
Urea	500 mg/dl

15.0 REFERENCES

1. Dorfman, RI and Shipley , RA, ED: Androgens, New York,: John Wiley and Sons, 1956.
2. Horton R, Tait JF: Androstenedione production and interconversion rates measured in peripheral blood and studies on the possible site of conversion to testosterone. J.Clin Invest 45: 301-303, 1966.
3. Faiman C and Winter, JSD, Reyes, FI, Clin *Obstet Gynaecol,* **3**, 467 (1976).
4. Sizonenka, PC, *Pediatrician,* **14,** 191 (1987).
5. Cummings DC, Wall SR: Non sex hormone binding globulin bound testosterone as a marker for hyperandrogenism. J. Clin Endocrinol Metab. 61:873-876, 1985.
6. Lashansky, G, et. al., *J Clin Endocrinol Metab*,**58**, 674 (1991)
7. Tietz, NW, ED: Clinical Guide to Laboratory Tests, 3rd ed. Philadelphia, WA Saunders Co, 1995.
8. Smith SW: Free Testosterone, *AACC Endo*; **11**(3), 59-62 (1993).

Revision: 2 **Date: 2018-Oct-01** **DCO: 1315**
MP5375 **Product Code: 5375-300**

Size		96(A)	192(B)
Reagent (fill)	A)	1ml set	1ml set
	B)	1ml set	1ml set
	C)	1 (6ml)	2 (6ml)
	D)	1 plate	2 plates
	E)	1 (20ml)	1 (20ml)
	F)	1 (7ml)	2 (7ml)
	G)	1 (7ml)	2 (7ml)

For Orders and Inquires, please contact
Monobind Inc.
100 North Pointe Drive
Lake Forest, CA 92630 USA
Tel: +1 949.951.2665 Mail: info@monobind.com
Fax: +1 949.951.3539 Fax: www.monobind.com

IVD 2°-8° CE
EC REP CEpartner4U, Esdoornlaan 13 3951 DB Maarn, The Neatherlands www.cepartner4u.eu

Please visit our website to learn more about our products and services.

Glossary of Symbols
(EN 980/ISO 15223)