Total Testosterone EIA

RUO

AL-162-r

INTENDED USE

Total Testosterone Enzyme Immunoassay (EIA) Kit provides materials for the quantitative measurement of Testosterone in serum. This assay is intended for *Research Use only.*

SUMMARY AND EXPLANATION

Testosterone, (17-Hydroxy-4-androstene-3-one), a C19 steroid, is one of the most potent naturally secreted androgens[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 [2,3]. In males, testosterone levels increase during the last trimester of fetal life due to placental and fetal pituitary gonadotropin stimulation, decline postnatally, increase again 30-60 days postnatally, then decline to low levels in childhood [4-6]. At the onset of male puberty, increased secretion of pituitary gonadotropins leads to increased testicular production of testosterone [4-7]. In adult men, plasma testosterone levels show a circadian variation, with peak levels in the morning [8]. In women, there is a much smaller increase in serum testosterone levels in the last trimester of fetal life, followed by low levels through childhood, and only a small increase during puberty [4,6]. The control of testosterone secretion in females is not completely defined. Feedback inhibition of gonadotropin secretion by testosterone has been demonstrated [9]. Testosterone production rate in blood has been estimated at 0.34 mg/day in adult females and more than 20 times this amount in adult males [2,3,10]. Approximately 60% of blood testosterone is normally bound with high affinity to sex hormone-binding globulin (SHBG) [11]; of the remainder, all but 1-2% is loosely bound to albumin. Both the unbound and albumin-bound fractions may be bioactive, while SHBG effectively inhibits testosterone action. Approximately half of the blood testosterone is metabolized in the liver to androsterone, etiocholanolone and epiandrosterone; all relatively weak androgens [1,3]. Testosterone is also converted to the potent androgen, dihydrotestosterone (DHT), in certain target tissues. In males, testosterone, either directly or after conversion to DHT, normally functions in fetal Wolffian duct differentiation, development of male external genitalia, stimulation of spermatogenesis in the postpubertal male, stimulation of anabolic activity in a variety of tissues and, possibly, behavioral and psychological effects [10,12]. The function of the low testosterone levels in normal females is not well defined. In both sexes, testosterone contributes to the development of secondary sexual hair, acne, and scalp hair patterns. In prepubertal males, elevated testosterone levels are found in both gonadotropin-dependent (e.g. CNS tumors, idiopathic true precocious puberty) and independent (e.g. testotoxicosis, adrenal hyperplasia or adrenal tumor) precocious puberty [13]. Testosterone levels are also elevated in androgen receptor defects [10]. In adolescent and adult males, low testosterone levels are associated with various pathologic conditions, including primary hypogonadism (e.g. testicular dysgenesis, Klinefelter syndrome) and gonadotropin deficiency (e.g. idiopathic hypogonadotropic hypogonadism, Kallman syndrome, hypopituitarism). In females of all ages, elevated testosterone levels can be associated with a variety of virilizing conditions, including congenital adrenal hyperplasia, adrenal tumors, arrhenoblastoma, mixed-gonadal dysgenesis, polycystic ovarian disease, ovarian hyperthecosis [3]. Measurement of testosterone levels in the immediate postnatal period can aid in the differential diagnosis of ambiguous genitalia, while measurements before and after exogenous gonadotropin administration can help to detect functional testicular tissue in cryptorchidism and other structural abnormalities [10,14,15]. The AL-162 Testosterone Enzyme immunoassay uses a sensitive and specific monoclonal anti-human testosterone and 11-oxy testosterone antibody. Although cross-reactivity occurs with DHT and a small number of androgen metabolites, the relative concentrations of these compounds in normal human samples predict that they will have a minimal effect on assay results.

PRINCIPLE OF THE TEST

The Total Testosterone EIA Kit uses the competitive binding enzyme immunoassay format. In the assay, Calibrators, Controls and Unknowns containing Testosterone are incubated with antibody labeled with enzyme horseradish peroxidase (HRP) and mouse anti-testosterone antiserum in microtitration wells coated with goat anti-mouse gamma globulin. After incubation and washing, the wells are incubated with the substrate tetramethylbenzidine (TMB). An acidic stopping solution is then added and the degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 and 630 nm.

MATERIALS SUPPLIED

CAL-162A - CAL-162F Testosterone Calibrators (Lyophilized)

1 mL labeled Calibrator A and 0.5 mL labeled B - F, containing concentrations of approximately 0 - 1500 ng/dL Testosterone in human serum matrix. Refer to **calibration card** for exact concentrations. **Calibrators are shipped ambient. Store at 2-8°C upon receipt until the expiration date.** Avoid repeated freeze thaws.

CTR-162-I and CTR-162- II Testosterone Controls (Lyophilized)

Two vials, 0.5 mL each, labeled Levels I and II containing low and high testosterone in Human serum matrix. Refer to **calibration card** for exact concentrations. **Controls are shipped ambient. Store at 2-8°C upon receipt until the expiration date**. Avoid repeated freeze thaws.

AG001 Goat Anti-Mouse IgG (GAMG) Microtitration Strips

One strip holder, containing 96 polystyrene microtitration wells with Goat anti-mouse IgG antibody immobilized to the inside wall of each well. Store at 2 to 8°C until expiration date in the resealable pouch with a desiccant to protect from moisture.

ECR-162 Testosterone Enzyme conjugate RTU

One bottle, 8.0 mL, containing a protein-based buffer with a non-mercury preservative. Store at 2 to 8°C until expiration date.

ABS-162 Anti-Testosterone Antibody Solution RTU

One bottle, 8 mL, containing anti-Testosterone mouse antibody in a protein-based buffer with a non-mercury preservative. Store at 2 to 8°C until expiration date.

TMB-100 TMB Chromogen Solution

One bottle, 12 mL, containing a solution of tetramethylbenzidine (TMB) in buffer with hydrogen peroxide. Store at 2 to 8°C until expiration date.

WSH-100 Wash Concentrate A

One bottle, 60 mL, containing buffered saline with a nonionic detergent. Store at 2 to 30°C until expiration date. Dilute 25-fold with deionized water prior to use.

STP-100 Stopping Solution

One bottle, 12 mL, containing 0.2 M sulfuric acid. Store at 2 to 30°C until expiration date.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Microtitration plate reader capable of absorbance measurement at 450 nm, 405nm and 630 nm.
2. Microtitration orbital plate shaker.
3. Microtitration plate washer.
4. Semi-automated/manual precision pipette to deliver 10–250 µL.
5. Repeater pipette
6. Repeater plus for repeated dispensing
7. Vortex mixer.
8. Deionized water.

WARNINGS AND PRECAUTIONS

For *Research Use Only*.

The following precautions should be observed:

a) Follow good laboratory practice.

b) Use personal protective equipment. Wear lab coats and disposable gloves when handling immunoassay materials.

c) Handle and dispose of all reagents and material in compliance with applicable regulations.

WARNING: Potential Biohazardous Material

This reagent may contain some human source material (e.g., serum) or materials used in conjunction with human source materials. Handle all reagents and patient samples at a Biosafety Level 2, as recommended for any potentially infectious human material in the Centers for Disease Control/National Institutes of Health manual "Biosafety in Microbiological and Biomedical Laboratories," 5th Edition, 2007[16].

WARNING: Potential Chemical Hazard

Some reagents in this kit contain Pro-Clean 400 and sodium azide as a preservative. Pro-Clean 400 and sodium azide in concentrated amounts are irritants to skin and mucous membranes.

For further information regarding hazardous substances in the kit, please refer to the MSDS, either at AnshLabs.com or by request.

SAMPLE COLLECTION AND PREPARATION

- Serum is the recommended sample type.
- Sample handling, processing, and storage requirements depend on the brand of blood collection tube that you use. Please reference the manufacturer's instructions for guidance. Each laboratory should determine the acceptability of its own blood collection tubes and serum separation products.
- Samples may be stored at 4°C if assayed within 7 days; otherwise, samples must be stored at -20°C or -80°C to avoid loss of bioactivity and contamination.
- Avoid assaying lipemic, hemolyzed or icteric samples.
- Avoid repeated freezing and thawing of samples. Thaw samples no more than 3 times.
- For shipping, place specimens in leak proof containers in biohazard specimen bags with appropriate specimen identification and test requisition information in the outside pocket of the biohazard specimen bag. Follow DOT and IATA requirements when shipping specimens[17].

PROCEDURAL NOTES

1. A thorough understanding of this package insert is necessary for successful use of the Total Testosterone EIA assay. It is the responsibility of the customer to validate the assay for their use. Accurate results will only be obtained by using precise laboratory techniques and following the package insert.
2. A calibration curve must be included with each assay.
3. Bring all kit reagents to room temperature before use. Thoroughly mix the reagents before use by gentle inversion. Do not mix different lots of any kit component and do not use any component beyond the expiration date.
4. Use a clean disposable pipette tip for each reagent, calibrator, control, or sample. Avoid microbial contamination of reagents, contamination of the substrate solutions with the SHRP conjugate. The enzyme used as the label is inactivated by oxygen, and is highly sensitive to microbial contamination, sodium azide, hypochlorous acid and aromatic chlorohydrocarbons often found in laboratory water supplies. Use deionized water.
5. Incomplete washing will adversely affect the outcome and assay precision. Care should be taken to add TMB into the wells accurately and efficiently to minimize potential assay drift due to variation in the TMB incubation time. Avoid exposure of the reagents to excessive heat or direct sunlight.

PREPARATION OF REAGENTS

1. **Wash Solution:** Dilute wash concentrate 25-fold with deionized water. The wash solution is stable for one month at room temperature when stored in a tightly sealed bottle.
2. **Microtitration Wells:** Select the number of coated wells required for the assay. The remaining unused wells should be placed in the resealable pouch with a desiccant. The pouch must be resealed to protect from moisture.

ASSAY PROCEDURE

Allow all specimens and reagents to reach room temperature (23 ± 2°C) and mix thoroughly by gentle inversion before use. Calibrators, controls, and unknowns should be assayed in duplicate.

NOTE: All serum samples reading higher than the highest calibrator should be mixed and diluted in the 0 ng/mL Calibrator A prior to assay.

1. Label the microtitration strips to be used.
2. Pipette **50 µL** of the **Calibrators, Controls, and unknowns** to the appropriate wells.
3. Add **50 µL** of **Testosterone Antibody Solution** to each well using a repeater pipette.
4. Add **50 µL** of **Testosterone Enzyme Conjugate Ready-To-Use** to each well using a repeater pipette.
5. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **60 minutes** at room temperature (23 ± 2°C).
6. Aspirate and wash each strip 5 times with Washing Solution (350 µL/per well) using an automatic microplate washer.
7. Add **100 µL** of the **TMB chromogen solution** to each well using a repeater pipette. Avoid exposure to direct sunlight.
8. Incubate the wells, shaking at **600–800 rpm** on an orbital microplate shaker, for **10-12 min** at room temperature (23 ± 2°C).
   NOTE: *Visually monitor the color development to optimize the incubation time.*
9. Add **100 µL** of the **Stopping solution** to each well using a repeater pipette. Read the absorbance of the solution in the wells within **20 minutes**, using a microplate reader set to **450 nm.**

NOTE: *If instrument has a wavelength correction, set the instrument to dual wavelength measurement at* ***450 nm*** *with background wavelength correction at* ***630 nm****.*

RESULTS

1. Optimum results can be obtained at incubation temperature of **23 ± 2°C**.
2. Calculate the mean OD for each calibrator, control, and sample.
3. Plot the mean OD readings for each of the Calibrators along the y-axis versus log of Testosterone calibrator concentrations in ng/dL on the x-axis, using a 4PL or sigmoid curve fit.
4. Determine the Total Testosterone concentrations of the controls and samples from the calibration curve by matching their mean OD readings with the corresponding Testosterone concentrations.
5. Any sample reading higher than the highest calibrator should be appropriately diluted into a low reading serum sample and re-assayed.
6. Any sample reading lower than the analytical sensitivity should be reported as such.

LIMITATIONS

The reagents supplied in this kit are optimized to measure Total Testosterone including the 11-keto testosterone levels in human serum matrix. If there is evidence of microbial contamination or excessive turbidity in a reagent, discard the vial. For assays employing antibodies, the possibility exists for interference by heterophile antibodies in the samples[18].

QUALITY CONTROL

- Each laboratory should establish mean values and acceptable ranges to assure proper performance.
- Testosterone EIA controls or other commercial controls should fall within established confidence limits.
- The confidence limits for Testosterone EIA controls are printed on the calibration card.
- A full calibration curve, low- and high-level controls, should be included in each assay.
- The TMB chromogen solution should be colorless. Development of a blue color may indicate reagent contamination or instability.

REPRESENTATIVE CALIBRATION CURVE DATA

Well Number	Well Contents Calibrators	Mean Absorbance	Conc (ng/dL)
A1, A2	A	2.5	0
B1, B2	B	2.2	9.0
C1, C2	C	1.5	40.0
D1, D2	D	0.9	140.0
E1, E2	E	0.3	500.0
F1, F2	F	0.1	1500.0

CAUTION: *The above data must not be employed in lieu of data obtained by the user in the laboratory.*

ANALYTICAL CHARACTERISTICS

All analytical characteristics are stated ng/dL.

Analytical Sensitivity:

The analytical sensitivity in the assay as calculated by the interpolation of mean plus two standard deviations of 16 replicates of calibrator A (0 ng/mL) and calibrator B (9.0 ng/dL) is 3.5 ng/dL.

Imprecision:

Reproducibility of the Total Testosterone EIA assay was determined in a study using samples in the low, mid, and high range. The study included a total of 14 assays, 3 replicates of each per assay (n=42). Representative data were calculated based on EP10A-3 protocol and are presented in the following table.

Sample	Mean conc.	Within run		Between run		Total	
	(ng/dL)	SD	%CV	SD	%CV	SD	%CV
Pool-1	104.6	5.9	5.6%	4.8	4.6%	7.5	7.2%
Pool-2	468.8	19.0	4.0%	26.5	5.7%	32.6	7.0%
Pool-3	1215.5	37.3	3.0%	101.7	8.4%	108.3	8.9%

Linearity:

Multiple dilutions of three samples containing various Testosterone levels were diluted in sample diluent (CAL-162A). The % recovery on individual samples is represented in the following table.

Sample ID	Dilution factor	Expected Conc. (ng/dL)	Observed Conc. (ng/dL)	%Recovery	Average %Recovery
F Dilution	NEAT	1500.0			102%
	2	750.0	785.1	105%	
	4	375.0	408.9	109%	
	8	187.5	203.8	109%	
	16	93.8	93.4	100%	
	32	46.9	42.3	90%	
S1	NEAT	528.3			110%
	2	264.1	276.4	105%	
	4	132.1	144.2	109%	
	8	66.0	77.0	117%	
	16	33.0	34.7	105%	
	32	16.5	18.7	113%	
S2	NEAT	345.4			110%
	2	172.7	169.2	98%	
	4	86.4	94.5	109%	
	8	43.2	51.2	119%	
	16	21.6	25.8	119%	
	32	10.8	11.6	107%	

Recovery:

Known amount of testosterone were added in five serum samples containing different levels of endogenous testosterone. The concentration of testosterone was determined before and after the addition of exogenous testosterone and the percent recovery was calculated.

Sample ID	Endogenous Conc. (ng/dL)	Expected Conc. (ng/dL)	Observed Conc. (ng/dL)	%Recovery
S1	100.5	162.5	154.8	95%
		218.9	202.4	92%
		317.5	298.4	94%
S2	80.9	143.9	128.6	89%
		201.1	168.0	84%
		301.3	263.6	87%
S3	369.9	419.1	389.0	93%
		463.8	410.8	89%
		542.1	455.8	84%
S4	36.6	101.7	86.1	85%
		160.8	140.1	87%
		264.4	233.4	88%
S5	472.8	517.1	514.5	99%
		557.4	535.3	96%
		627.9	579.8	92%

Analytical Specificity:

The table below summarizes results of cross-reactivity of proteins or compounds structurally and functionally related to testosterone in the Testosterone assay. The Total Testosterone assay detects Equine and Bovine Species.

Cross-Reactant	Concentration (ng/dL)	% Cross-Reactivity
Androstenedione	50 ng/mL	0.03%
Cortisol	50 ng/mL	0.04%
Ethynyl Estradiol	50 ng/mL	0.04%
Corticosterone	50 ng/mL	0.05%
Progesterone	50 ng/mL	0.06%
DHEA	50 ng/mL	0.05%
17-Hydroxy Progesterone	50 ng/mL	0.04%
Estriol	50 ng/mL	0.03%
Estrone	50 ng/mL	0.03%
DHT	50 ng/mL	5.2%
11-Keto Testosterone	50 ng/mL	27.1%

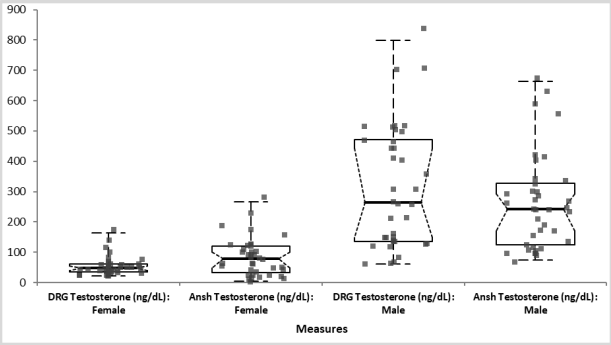
Interference:

When potential interferents (Hemoglobin, biotin, bilirubin, and intralipids) were added at least two times their physiological concentration to control sample, Testosterone concentration were within ± 25% of the control as represented in the following table. This study was based on NCCLS EP-7.

Interferent	Interferent Dose	Analyte Conc. (ng/dL)	Spiked Sample Value (ng/dL)	% Difference
Hemoglobin	1 mg/mL	260.6	291.0	11.7
	0.5 mg/mL	259.5	250.4	-3.5
	0.1 mg/mL	254.9	248.7	-2.4
Hemoglobin	1 mg/mL	257.7	232.2	-9.9
	0.5 mg/mL	249.4	250.0	0.2
	0.1 mg/mL	257.7	257.4	-0.1
Biotin	1200 ng/mL	285.8	291.0	1.8
	600 ng/mL	282.6	278.3	-1.5
	200 ng/mL	249.7	226.6	-9.2
Biotin	1200 ng/mL	236.3	237.6	0.6
	600 ng/mL	232.4	230.9	-0.7
	200 ng/mL	227.7	217.7	-4.4
Intralipids	20 mg/mL	230.0	246.0	7.0
	10 mg/mL	244.5	255.0	4.3
	5 mg/mL	239.4	246.6	3.0
Intralipids	20 mg/mL	235.1	244.7	4.1
	10 mg/mL	234.1	223.8	-4.4
	5 mg/mL	248.7	243.[illegible]	-2.1
Bilirubin	0.66 mg/mL	219.8	269.5	22.6
	0.2 mg/mL	254.7	265.9	4.4
Bilirubin	0.66 mg/mL	196.1	227.[illegible]	15.8
	0.2 mg/mL	220.7	231.[illegible]	5.1

Method Comparison:

Ansh Labs Total Testosterone ELISA (AL-162) has been compared to DRG Testosterone ELISA (EIA-1559) using 76 female and male serum samples. Distribution analysis of the results yielded the following as shown below.



Assay	N	Mean	Median
DRG Testosterone (ng/dL): Female	38	55.2	48.1
Ansh Total Testosterone (ng/dL): Female	38	84.2	78.3
DRG Testosterone (ng/dL): Male	38	314.0	264.0
Ansh Total Testosterone (ng/dL): Male	38	261.2	241.7

Hook Effect:

There is no high-dose hook effect at Testosterone concentrations up to 10,000 ng/dL.

Reference Ranges:

The expected ranges for Testosterone were calculated on 148 male serum samples and 84 Female serum samples using 95% non-parametric estimation using Analyse-It® for Microsoft Excel as shown as below.

Males

Age (Years)	No of specimens (n)	Median Conc. (ng/dL)	Testosterone (ng/dL) 95% CI
20-30	21	242.8	97.2 - 590.3
31-40	35	205.9	54.4 - 665.7
41-55	92	216.0	32.5 - 758.8

Females

Age (Years)	No of specimens (n)	Median Conc. (ng/dL)	Testosterone (ng/dL) 95% CI
21-35	27	76.5	11.3 - 669.4
36-55	57	63.3	5.1 - 667.1

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This assay is intended for ***Research Use Only***.

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