ab178663 – Free Testosterone ELISA Kit

Instructions for Use

A competitive immunoenzymatic assay for the quantitative measurement of Free Testosterone in serum and plasma (citrate).

This product is for research use only and is not intended for diagnostic use.

Version 4 Last Updated 4 February 2019

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1. BACKGROUND

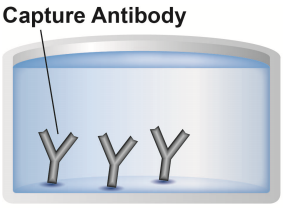
Abcam's Free Testosterone *in vitro* competitive ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the accurate quantitative measurement of Testosterone in serum and plasma.

A 96-well plate has been precoated with anti- Free Testosterone antibodies (solid-phase). Samples and the Free Testosterone-HRP conjugate are added to the wells, where Free Testosterone in the sample competes with the added Free Testosterone-HRP conjugate for antibody binding. After incubation, the wells are washed to remove unbound material and TMB substrate is then added which is catalyzed by HRP to produce blue coloration. The reaction is terminated by addition of Stop Solution which stops the color development and produces a color change from blue to yellow. The intensity of signal is inversely proportional to the amount of Free Testosterone in the sample and the intensity is measured at 450 nm.

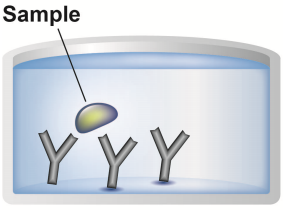
Testosterone is a steroid hormone from the androgen group. Testosterone is primarily secreted in the testes of males and the ovaries of females although small amounts are secreted by the adrenal glands. It is the principal male sex hormone and an anabolic steroid. In both males and females, it plays key roles in health and well-being.
Only a small percentage (< 1%) of circulating testosterone exists as unbound or free testosterone. The majority, approximately 60%, is bound to SHBG with high affinity, while the remainder is loosely bound to albumin. Both the albumin-bound and free fractions may be biologically active, while SHBG effectively inhibits testosterone action.
Testosterone effects can be classified as virilizing and anabolic effects. Anabolic effects include growth of muscle mass and strength, increased bone density and strength, and stimulation of linear growth and bone maturation. Virilizing effects include maturation of the sex organs.
Testosterone levels decline gradually with age in men.
Measurement of the free or unbound fraction of serum testosterone has been proposed as a means of estimating the physiologically bioactive hormone. Free testosterone levels are elevated in women with hyperandrogenism associated with hirsutism in the presence or

absence of polycystic ovarian disease. In addition, free testosterone measurements may be more useful than total testosterone in situations where SHBG is increased or decreased (e.g. hypothyroidism and obesity).

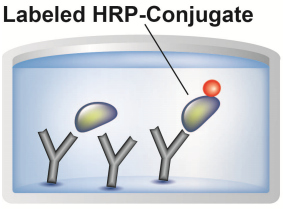
2. ASSAY SUMMARY



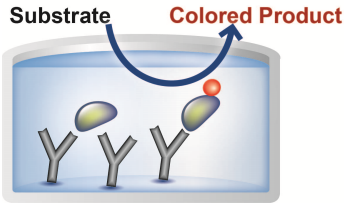
Prepare all reagents, samples, controls and standards as instructed.



Add samples, standards and controls to wells used.



Add prepared labeled HRP-Conjugate to each well. Incubate at 37°C.



After washing, add TMB substrate solution to each well. Incubate at room temperature. Add Stop Solution to each well. Read immediately.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at 2-8°C immediately upon receipt.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 9. Reagent Preparation.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
Anti-Testosterone IgG Coated Microplate (12 x 8 wells)	96 Wells	2-8°C
Stop Solution	15 mL	2-8°C
Testosterone HRP Conjugate	15 mL	2-8°C
TMB Substrate Solution	15 mL	2-8°C
Free Testosterone Control A	1 mL	2-8°C
Free Testosterone Control B	1 mL	2-8°C
10X Wash Solution	50 mL	2-8°C
Testosterone Standard 0 - 0 pg/mL	1 mL	2-8°C
Testosterone Standard 1 - 0.2 pg/mL	1 mL	2-8°C
Testosterone Standard 2 - 1.0 pg/mL	1 mL	2-8°C
Testosterone Standard 3 - 4.0 pg/mL	1 mL	2-8°C
Testosterone Standard 4 - 20 pg/mL	1 mL	2-8°C
Testosterone Standard 5 - 100 pg/mL	1 mL	2-8°C
Strip Holder	1 unit	2-8°C
Cover Foil	1 unit	2-8°C

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Microplate reader capable of measuring absorbance at 450 nm or 620 nm
- Incubator at 37°C
- Multi- and single-channel pipettes to deliver volumes between 10 and 1,000 µL
- Optional: Automatic plate washer for rinsing wells
- Rotating mixer
- Deionised or (freshly) distilled water.
- Disposable tubes
- Timer

7. LIMITATIONS

- ELISA kit intended for research use only. Not for use in diagnostic procedures
- All components of Human origin used for the production of these reagents have been tested for anti-HIV antibodies, anti-HCV antibodies and HBsAg and have been found to be non-reactive. Nevertheless, all materials should still be regarded and handled as potentially infectious
- Use only clean pipette tips, dispensers, and lab ware
- Do not interchange screw caps of reagent vials to avoid cross-contamination
- Close reagent vials tightly immediately after use to avoid evaporation and microbial contamination
- After first opening and subsequent storage check conjugate and control vials for microbial contamination prior to further use
- To avoid cross-contamination and falsely elevated results pipette patient samples and dispense conjugate, without splashing, accurately to the bottom of wells.

8. TECHNICAL HINTS

- If using automated equipment, the user has the responsibility to make sure that the kit has been appropriately tested. To improve the performance of the kit on ELISA automatic systems, it is recommended to increase the number of washes.
- Avoid foaming or bubbles when mixing or reconstituting components
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions
- Ensure plates are properly sealed or covered during incubation steps
- Complete removal of all solutions and buffers during wash steps is necessary for accurate measurement readings
- Addition of the TMB Substrate solution initiates a kinetic reaction, which is terminated by the addition of the Stop Solution. Therefore, the TMB Substrate and the Stop Solution should be added in the same sequence to eliminate any time deviation during the reaction
- It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond ten minutes to avoid assay drift. If more than 10 minutes are needed, follow the same order of dispensation. If more than one plate is used, it is recommended to repeat the dose response curve in each plate
- The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or increase the background
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions**

9. REAGENT PREPARATION

Equilibrate all reagents, samples and controls to room temperature (18-25°C) prior to use for at least 30 minutes. At the end of the assay return reagents to recommended storage immediately, avoid long term storage at room temperature.

9.1 1X Washing Solution

Prepare 1X Washing Solution by diluting 10X Washing Solution with deionized water. To make 500 mL 1X Washing Solution combine 50 mL 10X Washing Solution with 450 mL deionized water. Mix thoroughly and gently. Diluted solution is stable for 30 days at 2-8°C. In the concentrated solution it is possible to observe the presence of crystals, in this case mix at room temperature until complete dissolution of crystals.

- All other solutions are supplied ready to use

10. SAMPLE COLLECTION AND STORAGE

- The determination of Free Testosterone can be performed in human serum or plasma. Store specimen at -20°C if the determination is not performed on the day of the sample collection. If samples are stored frozen, mix thawed samples well before testing. Avoid repeated freezing and thawing.

11. PLATE PREPARATION

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents
- Unused well strips should be returned to the plate packet and stored at 4°C
- For each assay performed, a minimum of 1 well must be used as a blank, omitting sample and conjugate from well addition
- For statistical reasons, we recommend each standard and sample should be assayed with a minimum of two replicates (duplicates).

12. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **Please read the test protocol carefully before performing the assay. Result reliability depends on strict adherence to the test protocol as described.**
- **If performing the test on ELISA automatic systems we recommend increasing the washing steps from three to five and the volume of washing solution from 300 µL to 350 µL to avoid washing effects.**
- **Assay all standards, controls and samples in duplicate.**
- **All controls (Testosterone Positive) must be included with each assay performed to determine test results.**

12.1. Prepare all reagents, working standards, and samples as directed in the previous sections.

12.2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4°C storage.

12.3. Add 20 µL standard, controls or sample into their respective wells. Add 100 µL Free Testosterone-HRP Conjugate to each well. Leave a blank well for substrate blank.

12.4. Cover wells with the foil supplied in the kit.

12.5. Incubate for 1 hour at 37°C.

12.6.When incubation has been completed, remove the foil, aspirate the content of the wells and wash each well three times with 300 µL Wash Buffer. Avoid overflows from the reaction wells. During each washing step, gently shake the plate for 5 seconds and remove excess solution by tapping the inverted plate on an absorbent paper towel. (If you use automated equipment, wash the wells at least 5 times.

Note: Washing is critical. Insufficient washing results in poor precision and falsely elevated absorbance values.

12.7. Add 100 µL TMB Substrate Solution into all wells.

12.8. Incubate for exactly 15 minutes at room temperature in the dark.

12.9. Add 100 µL Stop Solution into all wells in the same order and at the same rate as for the TMB Substrate Solution. Shake the microplate gently. Any blue color developed during the incubation turns into yellow.

12.10. Measure the absorbance of the sample at 450 nm against a reference wavelength of 620-630 nm or against blank within 5 minutes of addition of the Stop Solution.

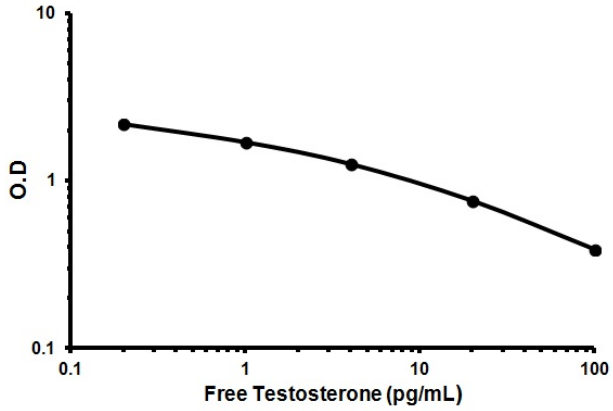
13. CALCULATIONS

Calculate the mean background subtracted absorbance for each point of the standard curve and each sample. Plot the mean value of absorbance of the standards against concentration. Draw the best-fit curve through the plotted points. (e. g.: Four Parameter Logistic).

Interpolate the values of the samples on the standard curve to obtain the corresponding values of the concentrations expressed in pg/mL.

14. TYPICAL SAMPLE VALUES

TYPICAL STANDARD CURVE -



REFERENCE VALUES-

The following values can be used as preliminary guideline until each laboratory has established its own normal range:

Group	Median	Mean (pg/mL) ± SD	Range (pg/mL)
Normal male	14	13 ± 7	4.5 – 42
Female Ovulating	1.3	1.4 ± 0.9	ND – 4.1
Female Oral contraceptives	0.9	1.1 ± 0.6	0.3 – 2.0
Female Postmenopausal	0.8	0.9 ± 0.5	0.1 – 1.7

SENSITIVITY –

Sensitivity was assessed by using 20 replicates of standard 0 and minimum 2 of standard 1. The lowest detectable concentration of Free Testosterone is 0.06 pg/mL.

PRECISION –

	Intra-Assay	Inter-Assay
n=	15	10
%CV	< 10	< 10

15. ASSAY SPECIFICITY

The specificity was assessed by measuring the apparent response of the assay to the following potentially cross-reactive analytes and interfering substances (Anticoagulants). The cross reaction of the antibody calculated at 50% is:

Testosterone	100.0 %
DHT	0.00008 %
Androstenedione	0.0043 %
Androsterone	0.00029 %
DHEA-S	0.00007 %
Cortisol	< 0.00001 %
Cortisone	< 0.00001 %
17 β Estradiol	0.00005 %
Estrone	< 0.00001 %
Prednisone	< 0.00001 %
17 α Ethynilestradiol	< 0.00001 %
Norgestrel	0.00001 %
Danazol	< 0.00001 %
Aldosterone	< 0.00001 %
Sodium citrate	< 0.00001 %
EDTA	< 0.00001 %
Heparin	< 0.00001 %

16. TROUBLESHOOTING

Problem	Cause	Solution
Low signal	Incubation time to short	Try overnight incubation at 4 °C
	Precipitate can form in wells upon substrate addition when concentration of target is too high	Increase dilution factor of sample
	Using incompatible sample type (e.g. serum vs. cell extract)	Detection may be reduced or absent in untested sample types
	Sample prepared incorrectly	Ensure proper sample preparation/dilution
Large CV	Bubbles in wells	Ensure no bubbles present prior to reading plate
	All wells not washed equally/thoroughly	Check that all ports of plate washer are unobstructed/wash wells as recommended
	Incomplete reagent mixing	Ensure all reagents/master mixes are mixed thoroughly
	Inconsistent pipetting	Use calibrated pipettes & ensure accurate pipetting
	Inconsistent sample preparation or storage	Ensure consistent sample preparation and optimal sample storage conditions (e.g. minimize freeze/thaws cycles)

Problem	Cause	Solution
High background	Wells are insufficiently washed	Wash wells as per protocol recommendations
	Contaminated wash buffer	Make fresh wash buffer
	Waiting too long to read plate after adding stop solution	Read plate immediately after adding stop solution
Low sensitivity	Improper storage of ELISA kit	Store all reagents as recommended. Please note all reagents may not have identical storage requirements.
	Using incompatible sample type (e.g. Serum vs. cell extract)	Detection may be reduced or absent in untested sample types

17. NOTES

UK, EU and ROW
Email: technical@abcam.com | Tel: +44-(0)1223-696000

Austria
Email: wissenschaftlicherdienst@abcam.com | Tel: 019-288-259

France
Email: supportscientifique@abcam.com | Tel: 01-46-94-62-96

Germany
Email: wissenschaftlicherdienst@abcam.com | Tel: 030-896-779-154

Spain
Email: soportecientifico@abcam.com | Tel: 911-146-554

Switzerland
Email: technical@abcam.com
Tel (Deutsch): 0435-016-424 | Tel (Français): 0615-000-530

US and Latin America
Email: us.technical@abcam.com | Tel: 888-77-ABCAM (22226)

Canada
Email: ca.technical@abcam.com | Tel: 877-749-8807

China and Asia Pacific
Email: hk.technical@abcam.com | Tel: 400 921 0189 / +86 21 2070 0500

Japan
Email: technical@abcam.co.jp | Tel: +81-(0)3-6231-0940

www.abcam.com | www.abcam.cn | www.abcam.co.jp