



SALIVARY TESTOSTERONE

ENZYME IMMUNOASSAY KIT

For Research Use Only

Item No. 1-2402, (Single) 96-Well Kit;
1-2402-5, (5-Pack) 480 Wells

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Must use IFU that is shipped with product.

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TABLE OF CONTENTS

Intended Use	1
Introduction	1
Test Principle	2
pH Indicator	2
Storage	2
Safety Precautions	2
Materials Supplied with Single Kit	3
Materials Needed but Not Supplied	4
Specimen Collection	4
Sample Handling and Preparation	5
Reagent Preparation	6
General Kit Use Advice	7
Procedure	8
Assay Summary	10
Calculations	11
Quality Control	11
Typical Results	12
Material Safety Information	13
Salivary Testosterone EIA Kit Performance Characteristics	13
Salivary Testosterone Expected Ranges	17
References	17
Warranty	19

Salivary Testosterone EIA Kit

Intended Use

The Salimetrics™ testosterone kit is a competitive immunoassay specifically designed and validated for the quantitative measurement of salivary testosterone. It is intended only for research use in humans and some animals.

Please read the complete kit insert before performing this assay. Failure to follow kit procedure and recommendations for saliva collection and sample handling may result in false values.

For further information about this kit, or the application, or the procedures in this insert, contact the technical service team at Salimetrics or your local sales representative.

Introduction

Testosterone is an anabolic steroid hormone synthesized from androstenedione in the Leydig cells of the testes of males and, in smaller quantities, in the ovaries of females. (1,2) Small amounts are also secreted by the adrenal glands in both sexes. (3) Testosterone production also occurs in peripheral tissues by conversion of circulating DHEA-S, DHEA, and androstenedione. (4) Testosterone exhibits a diurnal rhythm, with highest levels in the morning and a nadir around midnight. (4,5)

In men, testosterone plays an important role in the development of male reproductive tissues including the testes and prostate, as well as promoting secondary sexual characteristics such as increased muscle, bone mass, and hair growth. (6,7)

In blood, only 1-10% of testosterone is in its unbound or biologically active form. The remaining testosterone is bound to serum proteins. Unbound testosterone enters saliva via intracellular mechanisms, and in saliva the majority of testosterone is not protein-bound. Salivary testosterone levels are unaffected by salivary flow rate. (8) The serum-saliva correlation for testosterone is very high for males, but only modest for females, possibly because women's values often fall near the bottom of the measurable range for both serum and saliva immunoassay kits. (9,10)

This kit is designed to measure testosterone levels in saliva. The standard is in a saliva-like matrix. In addition, a built-in pH indicator warns the user of acidic or basic samples.

Test Principle

A microtitre plate is coated with rabbit antibodies to testosterone. Testosterone in standards and unknowns competes with testosterone linked to horseradish peroxidase for the antibody binding sites. After incubation, unbound components are washed away. Bound testosterone peroxidase is measured by the reaction of the peroxidase enzyme on the substrate tetramethylbenzidine (TMB). This reaction produces a blue color. A yellow color is formed after stopping the reaction using 2-molar sulfuric acid. Optical density is read on a standard plate reader at 450 nm. The amount of testosterone peroxidase detected is inversely proportional to the amount of testosterone present. (11)

pH Indicator

A pH indicator in the assay diluent alerts the user to samples with high or low pH values. Acidic samples will turn the diluent yellow. Alkaline samples will turn the diluent purple. Dark yellow or purple wells indicate that a pH value for that sample should be obtained using pH strips. Testosterone values from samples with a pH ≤ 4.0 or ≥ 9.0 may be artificially inflated or lowered. (12)

Storage

All components of this kit are stable at 2-8°C until the kit's expiration date.

Safety Precautions

- Liquid stop is a 2-molar solution of sulfuric acid. This solution is caustic; use with care.
- See “Material Safety Information” at the end of procedure.

Materials Supplied with Single Kit

	Item	Quantity/Size
1	Microtitre Plate Coated with polyclonal anti-testosterone antibodies.	1/96-well
2	Testosterone Standard 600 pg/mL. Serially dilute before use according to Reagent Preparation. Contains: testosterone, buffer, preservative.	1 vial/ 500 µL
3	Testosterone Controls High, Low. Ready to use. Contain: testosterone, buffer, preservative.	2 vials/500 µL each
4	Wash Buffer Concentrate (10x) Dilute before use according to Reagent Preparation. Contains: phosphate buffer, detergent, preservative.	1 bottle/100 mL
5	Testosterone Assay Diluent Contains: phosphate buffer, pH indicator, preservative.	1 bottle/60 mL
6	Testosterone Enzyme Conjugate Concentrate. Dilute before use with testosterone assay diluent. (See step 5 of Procedure.) Contains: testosterone conjugated to HRP, preservative.	1 vial/40-50 µL
7	TMB Substrate Solution Non-toxic, ready to use.	1 bottle/25 mL
8	2 M Stop Solution Contains: sulfuric acid.	1 bottle/12.5 mL
9	Non-Specific Binding (NSB) Wells Do not contain anti-testosterone antibody. Break off and insert as blanks (optional) where needed.	1 strip

Materials Needed But Not Supplied

- Precision pipette to deliver 18 μL , 25 μL , and 150 μL
- Precision multichannel pipette to deliver 50 μL , 150 μL , and 200 μL
- Vortex
- Plate rotator with 0.08-0.17 inch orbit (assay sensitivity may be affected if a rotator is not used)
- Plate reader with a 450 nm filter
- Computer software for data reduction
- Deionized water
- Reagent reservoirs
- One 20 mL disposable tube
- Five small disposable tubes
- Pipette tips
- 25 mL serological pipette

Specimen Collection

Avoid sample collection within 60 minutes after eating a major meal or within 12 hours after consuming alcohol. Acidic or high sugar foods can compromise assay performance by lowering sample pH and influencing bacterial growth. To minimize these factors, rinse mouth thoroughly with water 10 minutes before sample is collected.

Due to the episodic secretion pattern of steroid hormones, we can expect reproducible and reliable results only in cases of multiple sampling. Therefore, we recommend taking a minimum of 3 samples over a 2-hour period. Equal volumes from each of the samples should be pooled to create one sample that physically averages the fluctuations over that time period. (13,14)

Collecting whole saliva samples from adults and from children over 6 may be done by using the Salimetrics Oral Swab (SOS), Item no. 5001.02, or by unstimulated passive drool. Samples from children under the age of 6 may be collected with the Salimetrics Children's Swab (SCS), Item No. 5001.06. The Salimetrics Infant's Swab (SIS), Item No. 5001.08, is available for use with children under the age of 6 months.

Do not use Salivettes, Sorbettes, cotton, or polyester materials to collect samples. False readings will result. (15,16)

Samples visibly contaminated with blood should be recollected. Samples collected from populations that have little or no dental care, or known oral health problems, may be screened for possible blood contamination (17,18) using our Blood Contamination EIA Kit (Item nos. 1-1302/1-1312). Do not use dipsticks, which result in false positive values due to salivary enzymes.

Record the time and date of specimen collection.

Sample Handling and Preparation

After collection it is important to keep samples cold, in order to avoid bacterial growth in the specimen. Refrigerate sample within 30 minutes, and freeze at or below -20°C within 4 hours of collection. (Samples may be stored at -20°C or lower for long term storage.)

Do not add sodium azide to saliva samples as a preservative, as it may cause interference in the immunoassay.

Freezing saliva samples will precipitate the mucins. On day of assay, thaw completely, vortex, and centrifuge at $1500 \times g$ (@3000 rpm) for 15 minutes. Samples should be at room temperature before adding to assay plate. Pipette clear sample into appropriate wells. Be careful not to disrupt the pellet, since particulate matter may interfere with antibody binding, leading to falsely elevated results. It is important to avoid additional freeze-thaw cycles. However, if samples have been refrozen, centrifuge again prior to assaying.

Reagent Preparation

- Bring all reagents to room temperature and mix before use. A minimum of 1.5 hours is necessary for the 18 mL of assay diluent used in Step 5 (conjugate dilution) to come to room temperature.
- Bring microtitre plate to room temperature before use. ***It is important to keep the pouch with the plate strips closed until warmed to room temperature, as humidity may have an effect on the coated wells.***
- Prepare 1X wash buffer by diluting wash buffer concentrate 10-fold with room-temperature deionized water (100 mL of 10X wash buffer to 900 mL of deionized H₂O). ***Dilute only enough for current day's use, and discard any leftover reagent.*** (If precipitate has formed in the concentrated wash buffer, it may be heated to 40°C for 15 minutes. Cool to room temperature before use in assay.)
- Prepare serial dilutions of testosterone standard as follows:
 - Label five microcentrifuge tubes or other small tubes 2 through 6.
 - Pipette 90 µL of testosterone assay diluent into tubes 2 through 6.
 - Serially dilute the standard 2.5X by adding 60 µL of the 600 pg/mL standard (tube 1) to tube 2. Mix well. After changing pipette tips, transfer 60 µL from tube 2 to tube 3. Mix well. Continue for tubes 4, 5, and 6. The final concentrations of standards for tubes 1 through 6 are, respectively, 600 pg/mL, 240 pg/mL, 96 pg/mL, 38.4 pg/mL, 15.4 pg/mL, and 6.1 pg/mL. Standard concentrations in pmol/L are 2080.5, 832.2, 332.9, 133.2, 53.3, and 21.3, respectively.

General Kit Use Advice

- This kit uses break-apart microtitre strips. You may run less than a full plate. Unused wells must be stored at 2-8°C in the foil pouch with desiccant and used in the frame provided.
- The quantity of reagent provided with a single kit is sufficient for three partial runs. The volumes of wash buffer and conjugate prepared for assays using less than a full plate should be scaled down accordingly, keeping the same dilution ratio.
- Do not mix components from different lots of kits.
- When using a multichannel pipette, reagents should be added to duplicate wells at the same time. Follow the same sequence when adding additional reagents so that incubation time with reagents is the same for all wells.
- To ensure highest quality assay results, pipetting of samples and reagents must be done as quickly as possible (without interruption) across the plate. Ideally, the process should be completed within 20 minutes or less.
- When running multiple plates, or multiple sets of strips, a standard curve should be run with each individual plate and/or set of strips.
- The temperature of the laboratory may affect assays. Salimetrics' kits have been validated at 68-74°F (20-23.3°C). Higher or lower temperatures will cause an increase or decrease in OD values, respectively. Salimetrics cannot guarantee test results outside of this temperature range.
- Avoid microbial contamination of opened reagents. Salimetrics recommends using opened reagents within one month. Store all reagents at 2-8°C.
- Routine calibration of pipettes is critical for the best possible assay performance.

Procedure

Step 1: Determine your plate layout. Here is a suggested layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	600 Std	600 Std	Control High	Control High								
B	240 Std	240 Std	Control Low	Control Low								
C	96 Std	96 Std	Unknown 1	Unknown 1								
D	38.4 Std	38.4 Std	Unknown 2	Unknown 2								
E	15.4 Std	15.4 Std	Unknown 3	Unknown 3								
F	6.1 Std	6.1 Std	Unknown 4	Unknown 4								
G	Zero	Zero	Unknown 5	Unknown 5								
H	NSB	NSB	Unknown 6	Unknown 6								

Step 2: Keep the desired number of strips in the strip holder and place the remaining strips back in the foil pouch. If you choose to place non-specific binding wells in H-1, 2, remove strips 1 and 2 from the strip holder and break off the bottom wells. Place the strips back into the strip holder leaving H-1, 2 blank. Break off 2 NSB wells from the strip of NSBs included in the foil pouch. Place in H-1, 2. Alternatively, NSBs may be placed wherever you choose on the plate. Reseal the foil pouch with unused wells and desiccant. Store at 2-8°C.

Cautions: *1. Extra NSB wells should not be used for determination of standards, controls or unknowns.*

2. Do not insert wells from one plate into a different plate.

Step 3: Pipette 18 mL of testosterone assay diluent into the disposable tube. (Scale down proportionally if not using the entire plate.) Set aside for Step 5.

Step 4:

- Pipette 25 μL of standards, controls, and unknowns into appropriate wells. Standards, controls, and unknowns should be assayed in duplicate.
- Pipette 25 μL of testosterone assay diluent into 2 wells to serve as the zero.
- Pipette 25 μL of testosterone assay diluent into each NSB well.

Step 5: Dilute the enzyme conjugate 1:1000 by adding 18 μL of the conjugate to the 18 mL of testosterone assay diluent prepared in Step 3. (Scale down proportionally if not using the entire plate.) Immediately mix the diluted conjugate solution and add 150 μL to each well using a multichannel pipette.

Step 6: Mix plate on a plate rotator for 5 minutes at 500 rpm (or tap to mix) and incubate at room temperature for an additional 55 minutes.

Step 7: Wash the plate 4 times with 1X wash buffer. A plate washer is recommended. However, washing may be done by gently squirting wash buffer into each well with a squirt bottle, or by pipetting 300 μL of wash buffer into each well and then flipping the liquid into a sink. After each wash, the plate should be thoroughly blotted on paper towels before turning upright. If using a plate washer, blotting is still recommended after the final wash, just before the addition of the TMB.

Step 8: Add 200 μL of TMB solution to each well with a multichannel pipette.

Step 9: Mix on a plate rotator for 5 minutes at 500 rpm (or tap to mix) and incubate the plate in the dark at room temperature for an additional 25 minutes.

Step 10: Add 50 μL of stop solution with a multichannel pipette.

Step 11:

- Mix on a plate rotator for 3 minutes at 500 rpm (or tap to mix). Be sure all wells have turned yellow. If green color remains, continue mixing until green color turns to yellow.

Caution: *Spillage may occur if mixing speed exceeds 600 rpm.*

- Wipe off bottom of plate with a water-moistened, lint-free cloth and wipe dry.
- Read in a plate reader at 450 nm. Read plate within 10 minutes of adding stop solution. (For best results, a secondary filter correction at 490 to 630 nm is recommended.)

Assay Summary

1. Bring all reagents to room temperature and mix before use.
2. Bring plate to room temperature and prepare for use with NSB wells. (Use of NSB wells is optional.)
3. Prepare tube with 18 mL of testosterone assay diluent for conjugate dilution, which will be made later.
4. Prepare 1X wash buffer.
5. Serially dilute testosterone standard.
6. Pipette 25 μ L of standards, controls, and unknowns into appropriate wells.
7. Pipette 25 μ L of testosterone assay diluent into zero and NSB wells.
8. Make final 1:1000 dilution of conjugate (18 μ L into 18 mL assay diluent), mix, and immediately pipette 150 μ L into each well.
9. Mix for 5 minutes at 500 rpm. Incubate at room temperature for an additional 55 minutes.
10. Wash plate 4 times with 1X wash buffer. Blot.
11. Add 200 μ L TMB solution to each well.
12. Mix plate for 5 minutes at 500 rpm. Incubate in dark at room temperature for 25 additional minutes.
13. Add 50 μ L stop solution to each well. Mix for 3 minutes at 500 rpm.
14. Wipe plate bottom clean and read within 10 minutes of adding stop.

Calculations

1. Compute the average optical density (OD) for all duplicate wells.
2. Subtract the average OD for the NSB wells (if used) from the average OD of the zero, standards, controls, and unknowns (B).
3. Calculate the percent bound (B/Bo) for each standard, control, and unknown by dividing the average OD (B) by the average OD for the zero (Bo).
4. Determine the concentrations of the controls and unknowns by interpolation using software capable of logistics. We recommend using a 4-parameter sigmoid minus curve fit.
5. If a dilution of sample is used, multiply the assay results by the dilution factor. Samples with testosterone values greater than 600 pg/mL should be diluted with assay diluent and rerun for accurate results.

When running multiple plates, or multiple sets of strips, a standard curve should be run with each individual plate and/or set of strips.

Quality Control

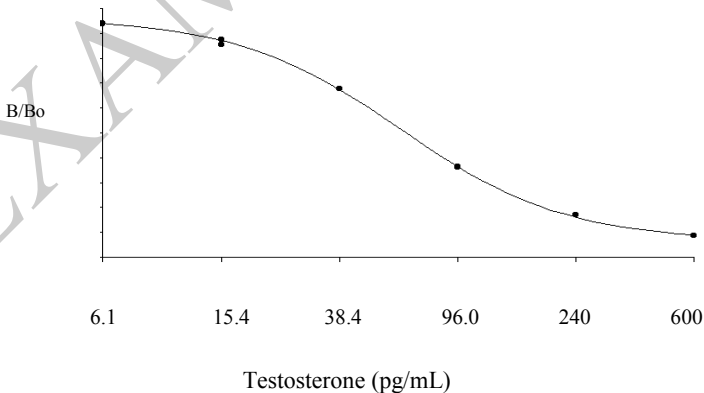
The Salimetrics' high and low salivary testosterone controls should be run with each assay. The control ranges established at Salimetrics are to be used as a guide. Each laboratory should establish its own range. Variations between laboratories may be caused by differences in techniques and instrumentation.

Typical Results

The following results are shown for illustration only and should not be used to calculate results from another assay.

Well	Sample	Average OD	B	B/Bo	Testosterone (pg/mL)
A1, A2	S1	0.225	0.203	0.088	600
B1, B2	S2	0.417	0.395	0.170	240
C1, C2	S3	0.863	0.841	0.362	96
D1, D2	S4	1.593	1.571	0.677	38.4
E1, E2	S5	2.026	2.004	0.864	15.4
F1, F2	S6	2.201	2.179	0.939	6.1
G1, G2	Bo	2.342	2.320	NA	NA
H1, H2	NSB	0.022	NA	NA	NA

Example: ER Testosterone 4-Parameter Sigmoid Minus Curve



Material Safety Information*

Hazardous Ingredients

Liquid stop solution is caustic; use with care. We recommend the procedures listed below for all kit reagents. Specific kit component MSDS sheets are available from Salimetrics upon request.

Handling

Follow good laboratory procedures when handling kit reagents. Laboratory coats, gloves, and safety goggles are recommended. Wipe up spills using standard absorbent materials while wearing protective clothing. Follow local regulations for disposal.

Emergency Exposure Measures

In case of contact, immediately wash skin or flush eyes with water for 15 minutes. Remove contaminated clothing. If inhaled, remove individual to fresh air. If individual experiences difficulty breathing, give oxygen and call a physician.

*The above information is believed to be accurate but is not all-inclusive. This information should only be used as a guide. Salimetrics shall not be liable for accidents or damage resulting from misuse of product.

Salivary Testosterone EIA Kit Performance Characteristics

Sensitivity

The lower limit of sensitivity was determined by interpolating the mean optical density minus 2 SDs of 10 sets of duplicates at the 0 pg/ml level. The minimal concentration of testosterone that can be distinguished from 0 is < 1.0 pg/ml.

Correlation

The correlation between saliva and total serum testosterone was determined by assaying 28 matched samples (15 adult males and 13 females). The saliva-serum correlation was $r(26) = 0.96$, $p < 0.001$. The saliva-serum correlation was stronger for males, $r = 0.91$, than for females, $r = 0.61$. (16) The relationship between serum and saliva for males as determined by linear regression is y (total serum testosterone in ng/mL) = $0.2421 + 0.0496 * x$ (salivary testosterone in pg/mL). The linear regression equation for females is y (total serum testosterone in ng/mL) = $0.1415 + 0.0055 * x$ (salivary testosterone in pg/mL).

Precision

1. The intra-assay precision was determined from the mean of 12 replicates each.

Sample	N	Mean (pg/ml)	Standard Deviation (pg/ml)	Coefficient of Variation (%)
H	12	188.83	4.69	2.5
L	12	18.12	1.22	6.7

2. The inter-assay precision was determined from replicates across 41 lots.

Sample	N	Mean (pg/ml)	Standard Deviation (pg/ml)	Coefficient of Variation (%)
H	64	199.08	11.18	5.6
L	63	19.6	2.69	14.05

Recovery

Saliva samples containing different levels of an endogenous testosterone were spiked with known quantities of testosterone and assayed.

Sample	Endogenous (pg/ml)	Added (pg/ml)	Expected (pg/ml)	Observed (pg/ml)	Recovery (%)
1	29.57	9.60	39.17	37.40	95.5
1	29.57	60.00	89.57	97.91	109.3
1	29.57	400.00	429.57	452.95	105.4
2	76.42	9.60	86.02	90.18	104.8
2	76.42	60.00	136.42	136.23	99.9
3	80.66	200.00	280.66	311.90	111.1

Specificity

The following compounds were tested at concentrations up to 1,000 ng/mL for cross-reactivity:

Compound	Spiked Concentration (ng/mL)	% Cross-reactivity
Aldosterone	1,000	ND
Androstenedione	10	1.157
Corticosterone	1,000	ND
Cortisol	1,000	ND
Cortisone	1,000	ND
11-Deoxycortisol	1,000	ND
21-Deoxycortisol	1,000	0.004
DHEA	1,000	ND
Dianabol	10	0.489
Dihydrotestosterone*	500	36.4
Epitestosterone	100	0.165
11-Hydroxytestosterone	10	1.90
19-Nortestosterone†	1000	21.02
Epitestosterone	100	0.165
Estradiol	51	0.025
Estriol	1,000	0.012
Estrone	1,000	0.005
Progesterone	1,000	0.005
17 α -Hydroxyprogesterone	1,000	ND
Transferrin	1,000	ND

ND = None detected (< 0.004)

*Literature states that salivary DHT levels expected in normal healthy adults, presenting no symptoms, is less than 10 pg/ml, well below the levels used to test cross reactivity. (19,20)

†Literature states that 19-nortestosterone is absent in normal healthy males & females, and that levels for pregnant females peak in the third trimester at 12-60 pg/ml, well below the levels used to test cross reactivity. (21)

Linearity of Dilution

Four saliva samples were serially diluted with testosterone assay diluent and assayed.

Sample	Dilution Factor	Expected (pg/ml)	Observed (pg/ml)	Recovery (%)
1			71.81	
	1:2	35.91	38.08	106
	1:4	17.95	19.31	107.6
	1:8	8.98	9.69	109
2			404.67	
	1:2	202.34	196.99	97.4
	1:4	101.17	94.12	93
	1:8	50.58	47.19	93.3
3			135.56	
	1:2	67.78	62.34	92
	1:4	33.89	35.86	105.8
	1:8	16.95	18.33	108.1
4			553.88	
	1:2	276.94	296.94	107.2
	1:4	138.47	141.01	101.8
	1:8	69.24	72.59	104.8
	1:16	34.62	38.55	111.4

Salivary Testosterone Expected Ranges*

Gender	N	Mean (pg/ml)	Median (pg/ml)	Range (5-95%)
Female	158	50.55	40.00	7.09-135.14
Male	87	165.50	136.18	59.05-335.12

Note: Early morning samples may be significantly higher.

*To be used as a guide only. Each laboratory should establish its own range.

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Seller's Limited Warranty

“Seller warrants that all goods sold hereunder will be free from defects in material and workmanship. Upon prompt notice by Buyer of any claimed defect, which notice must be sent within thirty (30) days from date such defect is first discovered and within three months from the date of shipment, Seller shall, at its option, either repair or replace the product that is proved to Seller's satisfaction to be defective. All claims should be submitted in written form. This warranty does not cover any damage due to accident, misuse, negligence, or abnormal use. Liability, in all cases, will be limited to the purchased cost of the kit.

It is expressly agreed that this limited warranty shall be in lieu of all warranties of fitness and in lieu of the warranty of merchantability. Seller shall not be liable for any incidental or consequential damages that arise out of the installation, use or operation of Seller's product or out of the breach of any express or implied warranties.”