



**Expanded Range**

# **SALIVARY TESTOSTERONE**

**ENZYME IMMUNOASSAY KIT**

For Research Use Only  
Not for use in Diagnostic Procedures

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



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## Intended Use

The Salimetrics® Testosterone Enzyme Immunoassay Kit is a competitive immunoassay specifically designed and validated for the quantitative measurement of salivary Testosterone. It is not intended for diagnostic use. It is intended only for research use in humans and some animals. Salimetrics has not validated this kit for serum or plasma samples.

***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 unreliable values.***

For further information about this kit, its application, or the procedures in this insert, please 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).



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## Test Principle

This is a competitive immunoassay kit. Testosterone in standards and samples compete with Testosterone conjugated to horseradish peroxidase for the antibody binding sites on a microtitre plate. After incubation, unbound components are washed away. Bound Testosterone Enzyme Conjugate is measured by the reaction of the horseradish peroxidase enzyme to the substrate tetramethylbenzidine (TMB). This reaction produces a blue color. A yellow color is formed after stopping the reaction with an acidic solution. The optical density is read on a standard plate reader at 450 nm. The amount of Testosterone Enzyme Conjugate detected is inversely proportional to the amount of Testosterone present in the sample (11).

## Safety Precautions

**Read Safety Data Sheets before handling reagents.**

### ***Hazardous Ingredients***

Liquid Stop Solution is caustic; use with care. We recommend the procedures listed below for all kit reagents.

### ***Handling***

Follow good laboratory practices when handling kit reagents. Laboratory coats, gloves, and safety goggles are recommended. Wipe up spills using appropriate 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 call a physician.

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

**Safety Data Sheets** are available by contacting Salimetrics at [support@salimetrics.com](mailto:support@salimetrics.com) (See [www.salimetrics.com](http://www.salimetrics.com) for alternative contact options).



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## 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.
- Avoid microbial contamination of opened reagents. Salimetrics recommends using opened reagents within one month. Store all reagents at 2-8°C.
- The quantity of reagent provided with a single kit is sufficient for three partial runs. The volumes of wash buffer and enzyme 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.
- 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 using a multichannel pipette to add reagents, always follow the same sequence when adding all reagents so that the incubation time is the same for all wells.
- When running multiple plates, or multiple sets of strips, a standard curve must 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 may affect OD values.
- Routine calibration of pipettes and other equipment is critical for the best possible assay performance.
- When mixing plates during assay procedures, avoid speeds that spill the contents of the wells.

## Storage

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

## pH Indicator

Testosterone values from samples with a pH  $\leq$  4.0 or  $\geq$  9.0 may be inaccurate. A pH indicator in the Testosterone Assay Diluent alerts the user to samples with high or low pH values. Upon addition of the Testosterone Assay Diluent, acidic samples will turn yellow and alkaline samples will turn purple. Dark yellow or purple wells indicate that a pH value for that sample should be obtained using pH strips. Samples with a pH  $\leq$  4.0 or  $\geq$  9.0 should be recollected (12).



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## 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.

Collect whole saliva by unstimulated passive drool. Donors may tilt the head forward, allowing the saliva to pool on the floor of the mouth, then pass the saliva through the SalivaBio Collection Aid (SCA) into a polypropylene vial. Collection protocols/methods are available online at [www.salimetrics.com](http://www.salimetrics.com) or upon request.

Samples visibly contaminated with blood should be recollected. Samples may be screened for possible blood contamination (13,14) using our Blood Contamination EIA Kit (Item Nos. 1-1302/1-1302-5). 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 for up to 6 months.) For long term storage, refer to the Salimetrics Collection and Handling Advice Booklet.

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

On day of assay, thaw the saliva samples completely, vortex, and centrifuge at 1500 x g for 15 minutes. Freezing saliva samples will precipitate mucins. Centrifuging removes mucins and other particulate matter which may interfere with antibody binding and affect results. Samples should be at room temperature before adding to assay plate. Pipette clear sample into appropriate wells. Re-freeze saliva samples as soon as possible after adding to the assay plate. Re-centrifuge saliva samples each time that they are thawed. Avoid multiple freeze-thaw cycles.



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## Materials Supplied with Single Kit

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



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## Materials Needed But Not Supplied

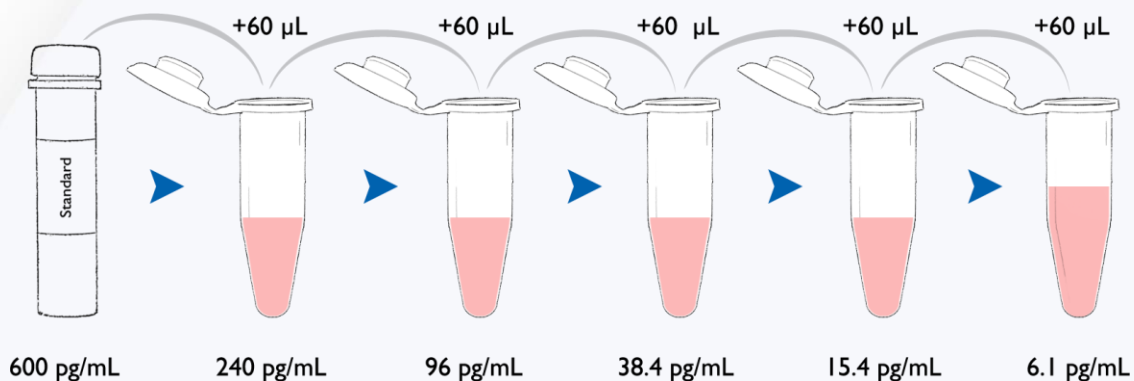
- Precision pipette to deliver 18  $\mu$ L, 25  $\mu$ L, and 150  $\mu$ L
- Precision multichannel pipette to deliver 50  $\mu$ L, 150  $\mu$ L, and 200  $\mu$ L
- Vortex
- Plate rotator with 0.08-0.17 inch orbit capable of 500 rpm
- Plate reader with 450 nm and 490 to 492 nm reference filters
- Computer software for data reduction
- Deionized water
- Reagent reservoirs
- One disposable polypropylene tube to hold at least 20 mL
- Five small disposable polypropylene tubes for dilution of standard
- Pipette tips
- Serological pipette to deliver up to 18 mL
- Centrifuge capable of 1500 x g





## Reagent Preparation

- Bring all reagents to room temperature and mix before use. A minimum of 1.5 hours is recommended for the 18 mL of Testosterone 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 foil 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 (10X) 10-fold with room-temperature deionized water (100 mL of Wash Buffer Concentrate (10X) to 900 mL of deionized water). ***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 the Testosterone Standard as follows:
  - Label five polypropylene microcentrifuge tubes or other small tubes 2 through 6.
  - Pipette 90  $\mu\text{L}$  of Testosterone Assay Diluent into tubes 2 through 6.
  - Serially dilute the standard 2.5X by adding 60  $\mu\text{L}$  of the 600 pg/mL standard (tube 1) to tube 2. Mix well.
  - After changing pipette tips, remove 60  $\mu\text{L}$  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.



## Procedure

**Step 1:** Read and prepare reagents according to the Reagent Preparation section before beginning assay. Determine your plate layout. Here is a suggested layout. (Standards, controls, and saliva samples should be assayed in duplicate.)

	1	2	3	4	5	6	7	8	9	10	11	12
A	600 Std	600 Std	Ctrl-H	Ctrl-H								
B	240 Std	240 Std	Ctrl-L	Ctrl-L								
C	96 Std	96 Std	SMP-1	SMP-1								
D	38.4 Std	38.4 Std	SMP-2	SMP-2								
E	15.4 Std	15.4 Std	SMP-3	SMP-3								
F	6.1 Std	6.1 Std	SMP-4	SMP-4								
G	Zero	Zero	SMP-5	SMP-5								
H	NSB*	NSB*	SMP-6	SMP-6								

\*NSB = Non-specific binding wells. These may serve as blanks. Use is optional.

**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 NSB wells 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 using less than the entire plate.) Set aside for Step 5.

### Step 4:

- Pipette 25 µL of standards, controls, and saliva samples into appropriate wells.
- 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.



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**Step 5:** Dilute the Enzyme Conjugate 1:1000 by adding 18  $\mu\text{L}$  of the conjugate to the 18 mL tube of Testosterone Assay Diluent. (Scale down proportionally if not using the entire plate.) Conjugate tube may be centrifuged for a few minutes to bring the liquid down to the tube bottom. Immediately mix the diluted conjugate solution and add 150  $\mu\text{L}$  to each well using a multichannel pipette.

**Step 6:** Mix plate on a plate rotator for 5 minutes at 500 rpm and incubate at room temperature for a total of 1 hour.

**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  $\mu\text{L}$  of wash buffer into each well and then discarding the liquid over 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 last wash.

**Step 8:** Add 200  $\mu\text{L}$  of TMB Substrate Solution to each well with a multichannel pipette.

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

**Step 10:** Add 50  $\mu\text{L}$  of Stop Solution with a multichannel pipette.

**Step 11:**

- Mix on a plate rotator for 3 minutes at 500 rpm. If green color remains, continue mixing until green color turns to yellow. Be sure all wells have turned 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 492 nm is recommended.)



## Quality Control

The Salimetrics' High and Low 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.

## 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 OD of the zero, standards, controls, and saliva samples.
3. Calculate the percent bound (B/Bo) for each standard, control, and saliva sample by dividing the OD of each well (B) by the average OD for the zero (Bo). (The zero is not a point on the standard curve.)
4. Determine the concentrations of the controls and saliva samples by interpolation using data reduction software. We recommend using a 4-parameter non-linear regression curve fit.
5. Samples with Testosterone values greater than 600 pg/mL should be diluted with Testosterone Assay Diluent and rerun for accurate results. If a dilution of the sample is used, multiply the assay results by the dilution factor.

***A new Standard Curve must be run with each full or partial plate.***

## Typical Results

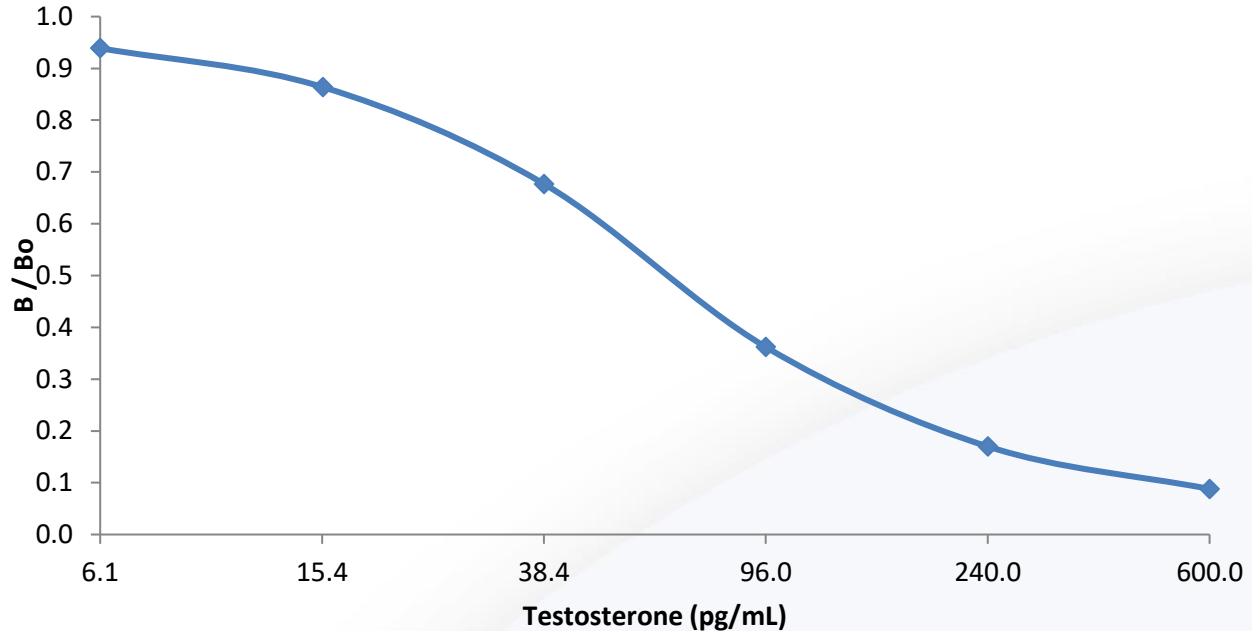
The results shown below are for illustration only and should not be used to calculate results from another assay.

Well	Standard	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



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## Example: Testosterone 4-Parameter Curve Fit



## Limitations

- Samples with Testosterone values greater than 600 pg/mL should be diluted with Testosterone Assay Diluent and rerun for accurate results. To obtain the final Testosterone concentration, multiply the concentration of the diluted sample by the dilution factor.
- A pH value should be obtained on samples that appear yellow or purple after the diluted conjugate solution is added and the plate is mixed (Step 6). Samples with pH values  $\leq 4.0$  or  $\geq 9.0$  should be recollected.
- See "Specimen Collection" recommendations to ensure proper collection of saliva specimens and to avoid interfering substances.
- Samples collected with sodium azide are unsuitable for this assay.
- Any quantitative results indicating abnormal Testosterone levels should be followed by additional testing and evaluation.



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## Salivary Testosterone Example Ranges\*

Female			
Age	Mean (pg/mL)	95% CI Range (pg/mL)	N
19-49	136.57	126.7-146.5	236
50-70	100.37	91.97-108.8	134

Male			
Age	Mean (pg/mL)	95% CI Range (pg/mL)	N
19-49	198.93	191.4-206.5	693
50-70	143.77	137-150.6	212

**Note:** Early morning samples may be significantly higher.

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

## Salivary Testosterone EIA Kit Performance Characteristics

### Precision

The intra-assay precision was determined from the means of 5 samples with 20 replicates of each.

Saliva Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	Coefficient of Variation (%)
I -1	20	340.37	22.97	6.7
I -2	20	123.04	2.32	1.9
I -3	20	66.13	3.27	4.9
I -4	20	26.72	1.58	5.9
I -5	20	11.85	0.79	6.7



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The inter-assay precision was determined from the mean of average duplicates for 5 samples run across two different lots.

Saliva Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	Coefficient of Variation (%)
1	20	346.23	20.75	6.0
2	20	117.28	5.18	4.4
3	20	67.27	3.97	5.9
4	20	29.25	2.40	8.2
5	20	15.04	1.95	13.0

### ***Recovery***

Three saliva samples containing different levels of an endogenous Testosterone were spiked with known quantities of Testosterone and assayed.

Saliva 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



## ***Sensitivity***

### **Analytical 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 0.458 pg/mL.

### **Functional Sensitivity**

The functional sensitivity is determined as the concentration of saliva samples (tested as 20 replicates and three separate runs) that results in the average lowest concentration with a Coefficient of Variation of 20% or less. The functional sensitivity of the Salivary ER Testosterone Assay is 0.68 pg/mL.

### ***Correlation with Serum***

The correlation between serum and saliva Testosterone was determined by assaying 29 matched samples using the Diagnostic Systems Laboratories Serum Testosterone Radioimmunoassay and the Salimetrics Salivary Testosterone EIA. (16 adult males and 13 females). The saliva-serum correlation was  $r(29) = 0.96$ ,  $p < 0.001$ . The saliva-serum correlation was stronger for males,  $r(16) = 0.91$ , than for females,  $r(13) = 0.62$ .

The relationship between serum and saliva for males as determined by linear regression is  $y$  (total serum Testosterone in ng/mL) =  $0.2421 + 0.0496 \cdot x$  (salivary Testosterone in pg/mL). The linear regression equation for females is  $y$  (total serum Testosterone in ng/mL) =  $0.1415 + 0.0055 \cdot x$  (salivary Testosterone in pg/mL).





## ***Sample Dilution Recovery***

Four samples were serially diluted with Testosterone Assay Diluent and assayed.

<b>Saliva Sample</b>	<b>Dilution Factor</b>	<b>Expected (pg/mL)</b>	<b>Observed (pg/mL)</b>	<b>Recovery (%)</b>
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
	1:16	25.29	24.52	97
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
	1:16	8.47	8.65	102.1
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

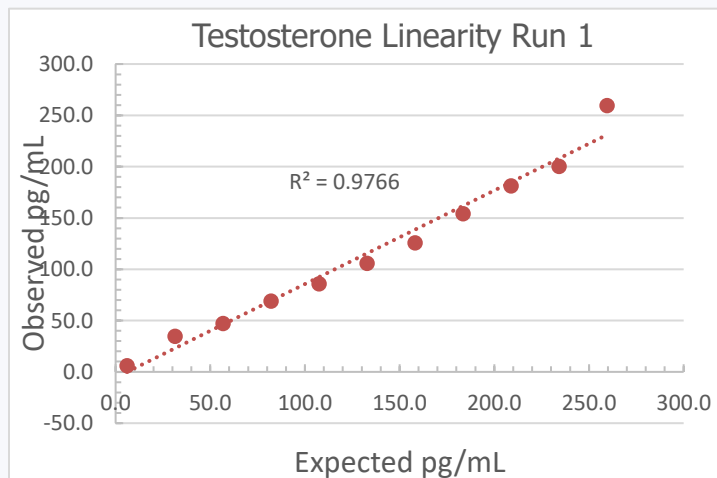


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## Linearity

Two saliva samples were diluted with each other proportionally and assayed.

Run 1						
Sample ID	Rep 1 pg/ml	Rep 2 pg/ml	Mean pg/ml	% CV	Expected	% Recovered
L1	264.16	254.89	259.52	3	259.52	100
L2	198.65	202.02	200.34	1	234.17	86
L3	181.33	181.33	181.33	0	208.82	87
L4	153.92	154.82	154.37	0	183.47	84
L5	126.40	125.40	125.90	1	158.13	80
L6	106.44	105.08	105.76	1	132.78	80
L7	85.99	85.60	85.79	0	107.43	80
L8	69.82	68.65	69.23	1	82.08	84
L9	47.71	46.55	47.13	2	56.73	83
L10	35.89	33.66	34.78	5	31.38	111
L11	6.14	5.92	6.03	3	6.03	100



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## Antibody Specificity

Compound	Spiked Concentration (ng/mL)	% Cross-reactivity in Salivary Testosterone EIA
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 $\alpha$ -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, are less than 10 pg/mL, well below the levels used to test cross reactivity (15,16).

†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 (17).



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## References

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