Anexos

Anexo 1 – Tabela do Atleta para o teste de Nado (tabela 1)

NOME: ______________________________ Nº ______________

DATA: ____________________________

	T Parcial	T Acumulado	FG
100m			
200m			
300m			
400m			
500m			
600m			
700m			
800m			
900m			
1000m			
1100m			
1200m			
1300m			
1400m			
1500m			
1600m			
1700m			
1900m			
2000m			

Lactato ______________

FC ______________

RPE ______________

DistânciaPercorrida

____

Anexo 2 - Protocolo de Recolha das Amostras de Saliva

PROTOCOLO DE RECOLHA DAS AMOSTRA DE SALIVA

As instruções em seguida apresentadas deverão ser cumpridas de forma a assegurar a fiabilidade dos dados recolhidos.

Em cada um dos dias de realização do estudo as recolhas de amostra de saliva serão realizadas em 6 momentos diferentes:

IDENTIFICAÇÃO DO MOMENTO	HORAS
A	19,00 horas
B	20,30 horas
C	21,30 horas
D	22,30 horas
E	Ao levantar (8-9 horas)
F	19,00 horas

Atenção:

☑ Antes de realizar a recolha das amostras de saliva os indivíduos não deverão ingerir alimentos, mastigar pastilhas elásticas ou rebuçados no período de 30 – 45 minutos que antecedem a recolha.

☑ Não se deverão lavar os dentes com pasta dentífrica antes das recolhas, sendo apenas permitido bocejar a boca com água.

☑ O tempo de recolha das amostra, onde cada indivíduo deverá mastigar o algodão, deverá ser de rigorosamente 2 minutos. Após o qual será colocado no recipiente próprio (tubo ensaio) e ir directamente para o congelador, mantendo-se lá até à sua recolha por parte dos investigadores.

A recolha das amostras no domicílio dos elementos da amostra será previamente combinada.

Em caso de qualquer dúvida, deverás contactar o investigador.

Contacto:

Obrigado, pela tua colaboração

Anexo 3 – Dados para caracterização da Amostra

Tabela 2 – Idade decimal, Massa, Estatura, IMC, Altura Sentado, Envergadura, ∑ Pregas, Anos de treino e Volume Médio Anual

Sujeitos	Idade Decimal (anos)	Massa (Kg)	Estatura (cm)	IMC	Altura Sentado (cm)	Envergadura (cm)	∑ Pregas	Anos de treino	Volume Médio Anual (Km)
01	15,79	55,20	164,50	20,40	84,00	171,00	44	8	1400
02	15,33	65,00	182,50	19,52	92,20	193,00	45	8	1400
03	16,93	58,00	174,00	19,16	87,00	184,00	41	7	1400
04	16,48	79,60	191,60	21,68	95,10	194,00	44	6	1400
05	17,64	59,40	177,40	18,87	91,50	190,00	32	6	1400
06	18,64	69,40	171,50	23,60	88,40	173,00	57	5	1400
07	17,44	59,80	172,60	20,07	91,50	174,00	59	8	1500
08	17,08	68,00	173,70	22,54	91,20	175,00	50	8	1500
09	17,65	72,00	183,00	21,50	95,00	190,00	41	9	1500
10	16,60	72,80	179,60	22,57	89,60	181,00	49	7	1500
11	17,50	68,20	182,60	20,45	92,50	187,00	36	7	1500
12	17,30	70,00	172,30	23,58	92,40	174,00	69	6	1500

Anexo 4 – Kit – Cortisol (Amostra e Controle)

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www.salimetrics.com
techservices@salimetrics.com

EXPANDED RANGE
High Sensitivity
SALIVARY CORTISOL ENZYME IMMUNOASSAY KIT

Catalog No. 1-3002/1-3012, 96-Well Kit (Contrato)

For Research Use

Intended Use

Salimetrics HS-Cortisol kit is a competitive immunoassay specifically designed for the quantitative measurement of salivary cortisol. It is not intended for use with serum/plasma or for diagnostic use. It is intended only for research use with saliva. Please read the complete kit insert before performing this assay. For further information about this kit, its application, or the procedures in this insert, please contact the technical service team at Salimetrics.

Introduction

Historically, the immunodiagnostic community's approach to the application of immunoassay techniques in the measurement of biomarkers in saliva has been problematic. This assay kit was designed to address those problems. First, prior to the late 1990s the majority of available immunoassays for saliva cortisol were modifications of protocols developed for the use with serum/plasma. The standards used in those assay kits were suspended in a human serum matrix. Given that the composition of serum is markedly different from saliva, those standards are likely to produce results that are influenced by matrix differences. To ensure the most accurate results, this salivary immunoassay uses a matrix that matches saliva. Second, the level of cortisol in saliva is significantly lower than levels in the general circulation. The use of a standard curve developed to capture the range of values expected in serum/plasma samples is often not sensitive enough to capture the complete range of individual differences in the level expected in saliva. This assay was designed to capture the full range of salivary cortisol levels (0.003 to 3.0 µg/dL) while using only 25 uL of saliva per test. Third, the pH of saliva is easily lowered or raised by the consumption of food or drink. Performance of immunoassays becomes compromised as the pH of samples to be tested drops below 4 (1). This results in artificially inflated levels. This assay system is designed to be resilient to the effects of interference caused by collection techniques that affect pH. In addition, a built-in pH indicator warns the user of acidic or basic samples.

Test Principle

A microtitre plate is coated with monoclonal antibodies to cortisol. Cortisol in standards and unknowns compete with cortisol linked to horseradish peroxidase for the antibody binding sites. After incubation, unbound components are washed away. Bound cortisol 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 with sulfuric acid. Optical density is read on a standard plate reader at 450 nm. The amount of cortisol peroxidase detected is inversely proportional to the amount of cortisol present (2).

Special Feature

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. Cortisol values from samples with a pH ≤ 3.5 or ≥ 9.0 may be artificially inflated or lowered (1).

Precautions

1. Stop Solution is a solution of sulfuric acid. This solution is caustic; use with care.
2. This kit uses break-apart microtitre strips. Unused wells must be stored at 2-8°C in the sealed foil pouch and used in the frame provided.
3. Do not mix components from different lots of kits.
4. 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.
5. See 'Material Safety Data' at the end of procedure.
6. As for all quantitative assays for salivary analytes, we recommend that samples be screened for possible blood contamination (3,4). This can be efficiently and economically accomplished using Salimetrics Blood Contamination EIA Kit (Cat No: 1-1302/1-1312). Do not use dipsticks, which result in false positive values due to salivary enzymes.
7. Routine calibration of pipettes is critical for the best possible assay performance.
8. Pipetting of samples and reagents must be done as quickly as possible (without interruption) across the plate.
9. When running multiple plates, or multiple sets of strips, a standard curve should be run with each individual plate and/or strips.
10. 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.

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

Reagents and Reagent Preparation

1. **Anti-Cortisol Coated Plate:** A ready-to-use 96-well microtitre plate pre-coated with monoclonal anti-cortisol antibodies in a resealable foil pouch.
2. **Cortisol Standards:** Six vials, 500 µL each, labeled A-F, containing cortisol concentrations of 3.000, 1.000, 0.333, 0.111, 0.037, and 0.012 µg/dL, in a synthetic saliva matrix with a non-mercury preservative. (Values in nmol/L are 82.77, 27.59, 9.19, 3.06, 1.02, and 0.33 nmol/L respectively.)
3. **Wash Buffer:** 100 mL of a 10X phosphate buffered solution containing detergents and a non-mercury preservative. Dilute the wash buffer concentrate 10-fold with room temperature deionized water (100 mL of 10X wash buffer to 900 mL of deionized H_2O). (NOTE: *If precipitate has formed in the concentrated wash buffer, it may be heated to 60°C for 15 minutes. Cool to room temperature before use in assay.)*
4. **Assay Diluent:** 63 mL of a phosphate buffered solution containing a pH indicator and a non-mercury preservative.
5. **Enzyme Conjugate:** 40 µL of a solution of cortisol labeled with horseradish peroxidase. Dilute prior to use with assay diluent.
6. **Tetramethylbenzidine (TMB):** 25 mL of a non-toxic ready to use solution.
7. **Stop Solution:** 12.5 mL of a solution of sulfuric acid in distilled water (US customers only). Stop solution is provided in powdered form to customers outside the US. Reconstitute the powdered stop solution with 12.5 mL of deionized water. Let sit for 10 minutes before use.
8. **Non-specific Binding Wells:** These wells do not contain anti-cortisol antibody. In order to support multiple-use, a strip of NSB wells is included. They are located in the foil pouch. Wells may be broken off and inserted where needed.

Note: The quantity of reagent provided with break-apart kits is sufficient for three individual runs. The volume of diluent and conjugate used for assays using less than a full plate should be scaled down accordingly, keeping the same dilution ratio.

Materials Needed But Not Supplied

- Precision pipette to deliver 15 and 25 µL
- Precision multichannel pipette to deliver 50 µL, and 200 µl
- Vortex
- Plate rotator (if unavailable, tap to mix)
- Plate reader with a 450 nm filter
- Log-linear graph paper or computer software for data reduction
- Deionized water
- Reagent reservoirs
- One disposable tube capable of holding 24 mL
- Pipette tips
- Serological pipette to deliver up to 24 mL

Precision:

1. The intra-assay precision was determined from the mean of 10 replicates each.

Sample	N	Mean (ug/dL)	Standard Deviation (ug/dL)	Coefficient of Variation (%)
H	10	0.897	0.01	3.88
M	10	0.51	0.03	6.22
L	10	0.14	0.01	7.12

2. The inter-assay precision was determined from the mean of average duplicates for ten separate runs.

Sample	N	Mean (ug/dL)	Standard Deviation (ug/dL)	Coefficient of Variation (%)
H	10	0.538	0.04	6.69
L	10	0.129	0.01	6.88

Linearity of Dilution: Three saliva samples were diluted with PBS and assayed.

Sample	Dilution Factor	Expected (ug/dL)	Observed (ug/dL)	Recovery (%)
1			0.513	
	1:2	0.256	0.271	105.8%
	1:4	0.128	0.134	104.7%
	1:8	0.064	0.057	89%
	1:16	0.032	0.036	112.5%
	1:32	0.016	0.015	93.8%
2			0.141	
	1:2	0.071	0.068	95.8%
	1:4	0.035	0.035	100%
	1:8	0.018	0.020	111.1%
3			0.387	
	1:2	0.193	0.199	103.1%
	1:4	0.097	0.100	103.1%
	1:8	0.048	0.054	112.5%
	1:16	0.024	0.023	95.8%
	1:32	0.012	0.011	91.7%

Sensitivity: The lower limit of sensitivity was determined by interpolating the mean minus 2SD for 10 sets of duplicates at 0 ug/dL standard. The minimal concentration of cortisol that can be distinguished from 0 is <.007 ug/dL.

Correlation with Serum: The correlation between serum and saliva cortisol was determined by assaying 19 matched samples using the Diagnostic Products Corporation serum Coat-a-Count Cortisol RIA and the Salimetrics HS Salivary Cortisol EIA.

The correlation between saliva and serum was highly significant, $\underline{r}$ (17) = 0.960, $\underline{p} < 0.0001$.

Normal Ranges: Refer to the Cortisol Diagnostic Kit Insert, #1-1102 on our web site.

Method Comparison: The correlation between the Salimetrics EIA and DPC's RIA Coat-a-Count Cortisol saliva modification was evaluated by assaying 72 common samples. The EIA-RIA results were highly correlated, $\underline{r}$ (72) = 0.956, $\underline{p} < 0.0001$.

Means: DPC RIA Cortisol = 0.309 μg/dL
Salimetrics EIA Cortisol = 0.322 μg/dL

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. This warranty does not cover any damage due to accident, misuse, negligence, or abnormal use.

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."

Procedure

Bring all reagents to room temperature.

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

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.80 Std	1.80 Std	Control H	Control H								
B	.600 Std	.600 Std	Control L	Control L								
C	.200 Std	.200 Std	Sample 1	Sample 1								
D	.067 Std	.067 Std	Sample 2	Sample 2								
E	.022 Std	.022 Std	Sample 3	Sample 3								
F	.007 Std	.007 Std	Sample 4	Sample 4								
G	Zero	Zero	Sample 5	Sample 5								
H	Nsb	Nsb	Sample 6	Sample 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 zip-lock and refrigerate the pouch at 2-8°C .
Caution: Extra NSB wells should not be used for determination of standards or unknowns.

Step 3:
- Pipette 24 mLs of assay diluent into a disposable tube. Set aside for Step 5.

Step 4:
- Pipette 25 µL of standards and unknowns into appropriate wells. Standards and samples should be assayed in duplicate.
- Pipette 25 µL of assay diluent into 2 wells to serve as the zero.
- Pipette 25 µL of assay diluent into each NSB well.

Step 5: Make a 1:1,600 dilution of the conjugate, by adding 15 µL of the conjugate to the 24 mL of assay diluent prepared in Step 3, (full plate only). Immediately mix the diluted conjugate solution and pipette 200 µL into each well using a multichannel pipette.

Step 6: Mix plate on 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 300µL of wash buffer into each well, and then discarding the liquid by inverting the plate over a sink. After each wash, the plate should be thoroughly blotted on paper towels before being turned upright. *If using a plate washer, blotting is still recommended after the last wash.*

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). **Caution:** *DO NOT mix at speeds over 600 rpms. Wells are very full!*
- 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 (correction at 492 to 620 is desirable).

Calculations

1. Compute the average Optical Density (OD) for all duplicate wells.
2. Subtract the average OD for the NSB wells from the average OD of the zero, standards, and unknowns.
3. Calculate the percent bound (B/BO) for each standard and unknown by dividing the average OD (B) by the average OD for the zero (BO).
4. If calculating the results by hand, plot B/BO on the vertical axis against the log of the concentration on the horizontal axis for each standard and draw a straight line through the points. Determine the concentrations of the unknowns by interpolation.
5. If using software capable of logistics, use a 4 parameter sigmoid minus curve fit. Otherwise, use log-linear regression.

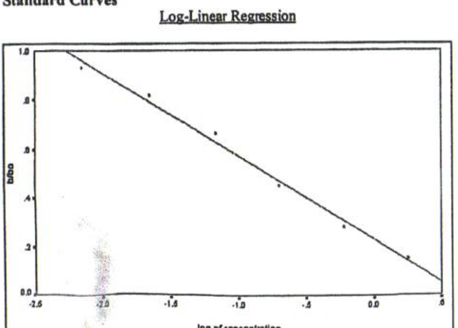
Typical Results

The following charts and graphs are for illustration only and **SHOULD NOT** be used to calculate results from another assay.

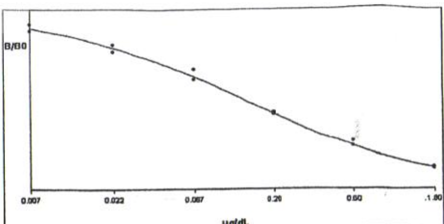
Well	Sample	Average OD	B	B/Bo	Cortisol ug/dL
A1,A2	S1	0.229	0.205	0.1207	1.613
B1,B2	S2	0.419	0.395	0.2326	0.757
C1,C2	S3	0.737	0.713	0.4199	0.214
D1,D2	S4	1.090	1.066	0.6278	0.052
E1,E2	S5	1.330	1.306	0.7691	0.020
F1,F2	S6	1.561	1.537	0.9052	0.008
G1,G2	B0	1.722	1.698	NA	
H1,H2	NSB	0.024	NA	NA	

Example: Standard Curves

Log-Linear Regression



Cortisol 4-Parameter Sigmoid Minus Curve Fit



Material Safety Data*

Hazardous Ingredients

Stop Solution is a solution of sulfuric acid. This solution is caustic; use with care. We recommend the procedures listed below for all kit reagents.

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 contact with reagents.

References

1. Schwartz, E.B., Granger, D.A., Susman, E.J., Gunnar, M.R., & Laird, B. (1998). Assessing salivary cortisol in studies of child development. *Child Development, 69,* 1503-1513.
2. Chard, T. (1990). *An introduction to radioimmunoassay and related techniques.* Amsterdam: Elsevier.
3. Kivlighan, K. T., Granger, D. A., Schwartz, E. B., Nelson, V., & Curran, M. (2004). Quantifying blood leakage into the oral mucosa and its effects on the measurement of cortisol, dehydroepiandrosterone, and testosterone in saliva. *Hormones and Behavior, 46,* 39-46.
4. Schwartz, E., & Granger, D. A. (2004). Transferrin enzyme immunoassay for quantitative monitoring of blood contamination in saliva. *Clinical Chemistry, 50,* 654-656.
5. Clements, A.D., & Parker, C.R. (1998). The relationship between salivary cortisol concentrations in frozen versus mailed samples. *Psychoneuroendocrinology, 23,* 613-616.
6. Kirschbaum, C., Read, G.F., & Hellhammer, D.H. (1992). *Assessment of hormones and drugs in saliva in biobehavioral research.* Kirkland, WA: Hogefe & Huber.

HS Cortisol EIA Assay Performance Characteristics

Recovery: Two saliva samples containing different levels of endogenous cortisol were spiked with known quantities of cortisol and assayed.

Sample	Endogenous (ug/dL)	Added (ug/dL)	Expected (ug/dL)	Observed (ug/dL)	Recovery (%)
1	0.41	0.54	0.95	0.825	86.8%
		0.04	0.450	0.390	86.7%
2	0.111	0.54	0.651	0.614	94.3%
		0.04	0.151	0.136	90.1%

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Catalog No. 1-3002/1-3012, 96-Well Kit (contrato)

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Salimetrics HS-Cortisol kit is a competitive immunoassay specifically designed for the quantitative measurement of salivary cortisol. It is not intended for use with serum/plasma or for diagnostic use. It is intended only for research use with saliva. Please read the complete kit insert before performing this assay. For further information about this kit, its application, or the procedures in this insert, please contact the technical service team at Salimetrics.

Introduction

Historically, the immunodiagnostic community's approach to the application of immunoassay techniques in the measurement of biomarkers in saliva has been problematic. This assay kit was designed to address those problems. First, prior to the late 1990s the majority of available immunoassays for saliva cortisol were modifications of protocols developed for the use with serum/plasma. The standards used in those assay kits were suspended in a human serum matrix. Given that the composition of serum is markedly different from saliva, those standards are likely to produce results that are influenced by matrix differences. To ensure the most accurate results, this salivary immunoassay uses a matrix that matches saliva. Second, the level of cortisol in saliva is significantly lower than levels in the general circulation. The use of a standard curve developed to capture the range of values expected in serum/plasma samples is often not sensitive enough to capture the complete range of individual differences in the level expected in saliva. This assay was designed to capture the full range of salivary cortisol levels (0.003 to 3.0 µg/dL) while using only 25 uL of saliva per test. Third, the pH of saliva is easily lowered or raised by the consumption of food or drink. Performance of immunoassays becomes compromised as the pH of samples to be tested drops below 4 (1). This results in artificially inflated levels. This assay system is designed to be resilient to the effects of interference caused by collection techniques that affect pH. In addition, a built-in pH indicator warns the user of acidic or basic samples.

Test Principle

A microtitre plate is coated with monoclonal antibodies to cortisol. Cortisol in standards and unknowns compete with cortisol linked to horseradish peroxidase for the antibody binding sites. After incubation, unbound components are washed away. Bound cortisol 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 with sulfuric acid. Optical density is read on a standard plate reader at 450 nm. The amount of cortisol peroxidase detected is inversely proportional to the amount of cortisol present (2).

Special Feature

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. Cortisol values from samples with a pH $\leq$ 3.5 or $\geq$ 9.0 may be artificially inflated or lowered (1).

Precautions

1. Stop Solution is a solution of sulfuric acid. This solution is caustic; use with care.
2. This kit uses break-apart microtitre strips. Unused wells must be stored at 2-8°C in the sealed foil pouch and used in the frame provided.
3. Do not mix components from different lots of kits.
4. 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.
5. See 'Material Safety Data' at the end of procedure.
6. As for all quantitative assays for salivary analytes, we recommend that samples be screened for possible blood contamination (3,4). This can be efficiently and economically accomplished using Salimetrics Blood Contamination EIA Kit (Cat No: 1-1302/1-1312). Do not use dipsticks, which result in false positive values due to salivary enzymes.
7. Routine calibration of pipettes is critical for the best possible assay performance.
8. Pipetting of samples and reagents must be done as quickly as possible (without interruption) across the plate.
9. When running multiple plates, or multiple sets of strips, a standard curve should be run with each individual plate and/or strips.
10. 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.

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

Reagents and Reagent Preparation

1. **Anti-Cortisol Coated Plate:** A ready-to-use 96-well microtitre plate pre-coated with monoclonal anti-cortisol antibodies in a resealable foil pouch.
2. **Cortisol Standards:** Six vials, 500 µL each, labeled A-F, containing cortisol concentrations of 3.000, 1.000, 0.333, 0.111, 0.037, and 0.012 µg/dL, in a synthetic saliva matrix with a non-mercury preservative. (Values in nmol/L are 82.77, 27.59, 9.19, 3.06, 1.02, and 0.33 nmol/L respectively.)
3. **Wash Buffer:** 100 mL of a 10X phosphate buffered solution containing detergents and a non-mercury preservative. Dilute the wash buffer concentrate 10-fold with room temperature deionized water (100 mL of 10X wash buffer to 900 mL of deionized H_20). (NOTE: *If precipitate has formed in the concentrated wash buffer, it may be heated to 60°C for 15 minutes. Cool to room temperature before use in assay.)*
4. **Assay Diluent:** 63 mL of a phosphate buffered solution containing a pH indicator and a non-mercury preservative.
5. **Enzyme Conjugate:** 40 µL of a solution of cortisol labeled with horseradish peroxidase. Dilute prior to use with assay diluent.
6. **Tetramethylbenzidine (TMB):** 25 mL of a non-toxic ready to use solution.
7. **Stop Solution:** 12.5 mL of a solution of sulfuric acid in distilled water (US customers only). Stop solution is provided in powdered form to customers outside the US. Reconstitute the powdered stop solution with 12.5 mL of deionized water. Let sit for 10 minutes before use.
8. **Non-specific Binding Wells:** These wells do not contain anti-cortisol antibody. In order to support multiple-use, a strip of NSB wells is included. They are located in the foil pouch. Wells may be broken off and inserted where needed.

Note: The quantity of reagent provided with break-apart kits is sufficient for three individual runs. The volume of diluent and conjugate used for assays using less than a full plate should be scaled down accordingly, keeping the same dilution ratio.

Materials Needed But Not Supplied

- Precision pipette to deliver 15 and 25 µL
- Precision multichannel pipette to deliver 50 µL, and 200 µl
- Vortex
- Plate rotator (if unavailable, tap to mix)
- Plate reader with a 450 nm filter
- Log-linear graph paper or computer software for data reduction
- Deionized water
- Reagent reservoirs
- One disposable tube capable of holding 24 mL
- Pipette tips
- Serological pipette to deliver up to 24 mL

Specimen Collection

The preferred saliva collection method (5,6) is to use plain (non-citric acid) cotton Salivettes (Sarstedt). Samples may also be collected using Sorbettes (for infants) or cotton ropes, or by passive drool. For accurate results collection devices should be completely saturated before removal. **Do not** add sodium azide to saliva samples as a preservative. Freeze at -20°C or lower for long-term storage. Contact the technical service team at salimetrics for more detailed information on specimen collection.

Saliva samples should be frozen prior to assay to precipitate the mucins. On day of assay, thaw completely, vortex, and centrifuge at 1500 x g (@3000 rpm) for 15 minutes. Pipette clear sample into appropriate wells. Particulate matter may interfere with antibody binding, leading to falsely elevated results. Do not assay samples that are visibly contaminated with blood.

Procedure

Bring all reagents to room temperature.

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

	1	2	3	4	5	6	7	8	9	10	11	12
A	3.000 Std	3.000 Std	Control H	Control H								
B	1.000 Std.	1.000 Std	Control L	Control L								
C	0.333 Std	0.333 Std	Sample 1	Sample 1								
D	0.111 Std	0.111 Std	Sample 2	Sample 2								
E	0.037 Std	0.037 Std	Sample 3	Sample 3								
F	0.012 Std	0.012 Std	Sample 4	Sample 4								
G	Zero	Zero	Sample 5	Sample 5								
H	NSB	NSB	Sample 6	Sample 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 zip-lock and refrigerate the pouch at 2-8°C .
Caution: Extra NSB wells should not be used for determination of standards or unknowns.

Step 3:

- Pipette 24 mL of assay diluent into a disposable tube. Set aside for Step 5.

Step 4:

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

Step 5: Make a 1:1600 dilution of the conjugate, by adding 15 μL of the conjugate to the 24 mL of assay diluent prepared in Step 3, (full plate only). Immediately mix the diluted conjugate solution and pipette 200 μL into each well using a multichannel pipette.

Step 6: Mix plate on 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 300μL of wash buffer into each well, and then discarding the liquid by inverting the plate over a sink. After each wash, the plate should be thoroughly blotted on paper towels before being turned upright. *If using a plate washer, blotting is still recommended after the last wash.*

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). **Caution**: _DO NOT_ *mix at speeds over 600 rpms.*
- 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 (correction at 492 to 620 is desirable).

Calculations

1. Compute the average optical density (OD) for all duplicate wells.
2. Subtract the average OD for the NSB wells from the average OD of the zero, standards, and unknowns.
3. Calculate the percent bound (B/Bo) for each standard and unknown by dividing the average OD (B) by the average OD for the zero (Bo).
4. If calculating the results by hand, plot B/Bo on the vertical axis against the log of the concentration on the horizontal axis for each standard and draw a straight line through the points. Determine the concentrations of the unknowns by interpolation.
5. If using software capable of logistics, use a 4-parameter sigmoid minus curve fit. Otherwise, use log-linear regression.

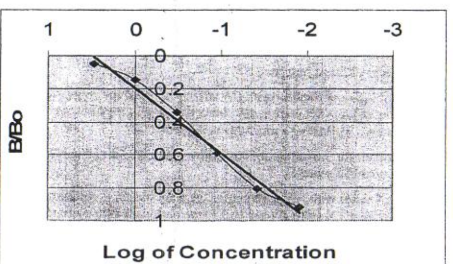
Typical Results

The following charts and graphs are for illustration only and SHOULD NOT be used to calculate results from another assay.

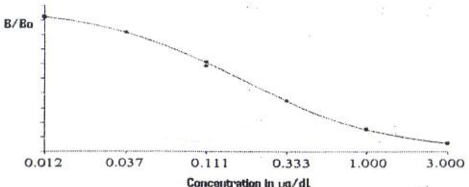
Well	Sample	Average OD	B	B/Bo	Cortisol (µg/dL)
A1,A2	S1	0.094	0.071	0.048	3.000
B1,B2	S2	0.236	0.213	0.145	1.000
C1,C2	S3	0.524	0.501	0.340	0.333
D1,D2	S4	0.897	0.874	0.593	0.111
E1,E2	S5	1.219	1.196	0.812	0.037
F1,F2	S6	1.379	1.356	0.921	0.012
G1,G2	Bo	1.496	1.473	NA	NA
H1,H2	NSB	0.023	NA	NA	NA

Example: Standard Curves

Log-Linear Regression



Cortisol 4-Parameter Sigmoid Minus Curve Fit



Material Safety Data*

Hazardous Ingredients

Stop Solution is a solution of sulfuric acid. This solution is caustic; use with care. We recommend the procedures listed below for all kit reagents.

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 contact with reagents.

HS Cortisol EIA Assay Performance Characteristics

Recovery: Six saliva samples containing different levels of endogenous cortisol were spiked with known quantities of cortisol and assayed.

Sample	Endogenous (µg/dL)	Added (µg/dL)	Expected (µg/dL)	Observed (µg/dL)	Recovery (%)
1	0.088	2.000	2.088	2.176	104.2
2	0.077	0.300	0.377	0.380	100.8
3	0.062	0.011	0.073	0.071	97.3
4	0.066	2.500	2.566	2.723	106.1
5	0.210	0.330	0.510	0.508	99.6
6	0.086	0.011	0.097	0.094	96.9

Precision:

1. The intra-assay precision was determined from the mean of 14 (low) and 18 (high) replicates each.

Sample	N	Mean (µg/dL)	Standard Deviation (µg/dL)	Coefficient of Variation (%)
Level 1	18	0.999	0.033	3.35
Level 2	14	0.097	0.004	3.65

2. The inter-assay precision was determined from the mean of average duplicates for 12 separate runs.

Sample	N	Mean (µg/dL)	Standard Deviation (µg/dL)	Coefficient of Variation (%)
Level 1	12	1.020	0.038	3.75
Level 2	12	0.101	0.006	6.41

Linearity of Dilution: Two saliva samples were diluted with PBS and assayed.

Sample	Dilution Factor	Expected (µg/dL)	Observed (µg/dL)	Recovery (%)
1			2.176	
	1:2	1.088	1.065	97.9
	1:4	0.544	0.503	92.5
	1:8	0.272	0.233	85.7
	1:16	0.136	0.109	80.1
2			0.508	
	1:2	0.254	0.247	97.2
	1:4	0.127	0.118	92.9
	1:8	0.064	0.058	90.6
	1:16	0.032	0.031	96.9

Sensitivity: The lower limit of sensitivity was determined by interpolating the mean minus 2 SD for 10 sets of duplicates at 0 µg/dL standard. The minimal concentration of cortisol that can be distinguished from 0 is < 0.003 µg/dL.

Correlation with Serum: The correlation between serum and saliva cortisol was determined by assaying 49 matched samples using the Diagnostic Systems Laboratories' serum Cortisol EIA and the Salimetrics ER HS Salivary Cortisol EIA.

The correlation between saliva and serum was highly significant, $r(47) = 0.91$, $p < 0.0001$.

References

1. Schwartz, E.B., Granger, D.A., Susman, E.J., Gunnar, M.R., & Laird, B. (1998). Assessing salivary cortisol in studies of child development. *Child Development, 69,* 1503-1513.
2. Chard, T. (1990). *An introduction to radioimmunoassay and related techniques.* Amsterdam: Elsevier.
3. Kivlighan, K. T., Granger, D. A., Schwartz, E. B., Nelson, V., & Curran, M. (2004). Quantifying blood leakage into the oral mucosa and its effects on the measurement of cortisol, dehydroepiandrosterone, and testosterone in saliva. *Hormones and Behavior, 46,* 39-46.
4. Schwartz, E., & Granger, D. A. (2004). Transferrin enzyme immunoassay for quantitative monitoring of blood contamination in saliva. *Clinical Chemistry, 50,* 654-656.
5. Clements, A.D., & Parker, C.R. (1998). The relationship between salivary cortisol concentrations in frozen versus mailed samples. *Psychoneuroendocrinology, 23,* 613-616.
6. Kirschbaum, C., Read, G.F., & Hellhammer, D.H. (1992). *Assessment of hormones and drugs in saliva in biobehavioral research.* Kirkland, WA: Hogefe & Huber.

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 writing. 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."

Anexo 5 – Kit - Testosterona

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SALIVARY TESTOSTERONE ENZYME IMMUNOASSAY KIT

Catalog No. 1-1402/1-1412, 96-Well Kit

For *in vitro* Research Use

Intended Use

Salimetrics' testosterone kit is a competitive immunoassay specifically designed for the quantitative measurement of salivary testosterone. It is not intended for use with serum/plasma or for diagnostic use. It is intended for research use with saliva. Please read the complete kit insert before performing this assay. For further information about this kit, its application, or the procedures in this insert, contact the technical service team at Salimetrics.

Introduction

Measurement of hormones in saliva has excited interest because of numerous potential applications in developmental and health-oriented behavioral studies (1). Although salivary assays for some hormones (e.g., cortisol) are widely available and used, the availability and use of salivary testosterone has been more restricted. A recent paper (2) reveals a need for a sensitive, efficient, reliable, and commercially available assay system for the measurement of salivary testosterone. This assay kit fills that need with a protocol that the research community can use to improve the next generation of their studies. It has been designed to specifically address the following five issues. First, all available immunoassays for salivary testosterone are modifications of protocols developed for the use with serum/plasma. The standards used in those assay kits are suspended in a human serum matrix. Given that the composition of serum is markedly different from saliva, these standards are likely to produce results that are influenced by matrix differences. To ensure the most accurate results, this salivary immunoassay is designed using a matrix that matches saliva. Second, the level of testosterone in saliva (pg/mL) is significantly lower than levels in the general circulation (ng/mL). The use of a standard curve developed to capture the range of values expected in serum/plasma samples is often not sensitive enough to capture individual differences in the level expected in saliva. This assay has been designed to do so. Third, the test volume of published modified serum immunoassays for use with salivary testosterone ranges from 200 µL to 1 mL per test. The current protocol uses only 50 µL of saliva per test. Fourth, no separation or extractions are necessary. Fifth, the pH of saliva is easily lowered or raised by the consumption of food or drink. Immunoassay performance can be compromised as the pH of samples to be tested drops below 4 (3). This assay system is designed to be very sensitive to the effects of interference caused by collection techniques that affect pH.

Test Principle

A microtitre plate is coated with rabbit antibodies to testosterone. Testosterone in standards and unknowns compete 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 with 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 (4).

Revision Date: 10-14-05

Special Feature

As in all Salimetrics assays for use with saliva, 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 a pH strip/meter. Testosterone values from samples with a pH ≤ 4.0 or ≥ 9.0 may be artificially inflated or lowered (3).

Precautions

1. Stop Solution is a 2 molar solution of sulfuric acid. This solution is caustic; use with care.
2. This kit uses break-apart microtitre strips. Unused wells must be stored at 2-8°C in the sealed foil pouch and used in the frame provided.
3. Do not mix components from different lots of kits.
4. 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.
5. See 'Material Safety' Data at the end of procedure.
6. We recommend that samples be screened for possible blood contamination (5,6) using a reliable screening tool such as the Salimetrics Blood Contamination EIA Kit (Cat No: 1-1302/1-1312). Do not use dipsticks, which result in false positive values due to salivary enzymes.
7. Routine calibration of pipettes is critical for the best possible assay performance.
8. Pipetting of samples and reagents must be done as quickly as possible (without interruption) across the plate.
9. When running multiple plates, or multiple sets of strips, a standard curve should be run with each individual plate and/or strips.
10. 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.

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

Reagents and Reagent Preparation

1. **Anti-Testosterone Coated Plate:** A ready-to-use 96-well microtitre plate pre-coated with rabbit anti-testosterone antibodies in a resealable foil pouch.
2. **Testosterone Standard:** 1 mL of testosterone in a in a saliva-like matrix with a non-mercury preservative, at a concentration of 360 pg/mL.
3. **Wash Buffer:** 100 mL of a 10X phosphate buffered solution containing detergents and a non-mercury preservative. Dilute the wash buffer concentrate 10-fold with room temperature deionized water (100 mL of 10X wash buffer to 900 mL of deionized H_20). (NOTE: *If precipitate has formed in the concentrated wash buffer, it may be heated to 60°C for 15 minutes. Cool to room temperature before use in assay.)*
4. **Assay Diluent:** 63 mL of a phosphate buffered solution containing a pH indicator and a non-mercury preservative.
5. **Enzyme Conjugate:** 50 µL of a solution of testosterone labeled with horseradish peroxidase. Dilute prior to use with assay diluent.
6. **Tetramethylbenzidine (TMB):** 25 mL of a non-toxic ready-to-use solution.
7. **Stop Solution:** 12.5 mL of a 2 molar solution of sulfuric acid in distilled water (US customers only). Stop solution is provided in powdered form to customers outside the US. Reconstitute the powdered stop solution with 12.5 mL of deionized water. Let sit for 10 minutes before use.
8. **Non-specific Binding Wells:** These wells do not contain anti-testosterone antibody. In order to support multiple-use, a strip of NSB wells is included. They are located in the foil pouch. Wells may be broken off and inserted where needed.

NOTE: *The quantity of reagent provided with break-apart kits is sufficient for three individual runs. The volume of diluent and conjugate used for assays using less than a full plate should be scaled down accordingly, keeping the same dilution ratio.*

Materials Needed But Not Supplied

- Precision pipette to deliver 7 µL, 50 µL, 100 µL and 200 µL
- Precision multichannel pipette to deliver 50 µL, 150 µL, and 200 µL
- Vortex
- Plate rotator (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

The preferred saliva collection method is by unstimulated passive drool. **Do not use any cotton absorbent material, such as Salivettes®, Sorbettes and cotton ropes or swabs to collect samples (1,7), as false high readings will result.** Do not use polyester versions of the Salivette device, or the Salisaver device. **Do not** add sodium azide to saliva samples as a preservative. Freeze at -20^{0}C or lower for long-term storage. Contact the technical service team at salimetrics for more detailed information on specimen collection.

Saliva samples should be frozen prior to assay to precipitate the mucins. On day of assay, thaw completely, vortex, and centrifuge at 1500 x g (@3000 rpm) for 15 minutes. It is important to avoid additional freeze-thaws cycles. Pipette clear sample into appropriate wells. Particulate matter may interfere with antibody binding, leading to falsely elevated results.

Procedure

Bring all reagents to room temperature.

Step 1: Determine your plate layout (see below).

	1	2	3	4	5	6	7	8	9	10	11	12
A	360 Std	360 Std	Ctrl H	Ctrl H								
B	144 Std	144 Std	Ctrl L	Ctrl L								
C	57.6 Std	57.6 Std	S 1	S 1								
D	23.0 Std	23.0 Std	S 2	S 2								
E	9.2 Std	9.2 Std	S 3	S 3								
F	3.7 Std	3.7 Std	S 4	S 4								
G	Zero	Zero	S 5	S 5								
H	NSB	NSB	S 6	S 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 pouch and refrigerate at 2-8°C.

Caution: Extra NSB wells should not be used for determination of standards or unknowns.

Step 3:

- Label five microcentrifuge tubes or other small tubes 2 through 6.
- Pipette 150 µL of assay diluent in tubes 2 through 6.

  Serially dilute the standard 2.5X by adding 100 µL of the 360 pg/mL standard (tube 1) to tube 2. Mix well. After changing pipette tips, remove 100 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 respectively are 360 pg/mL, 144 pg/mL, 57.6 pg/mL, 23.0 pg/mL, 9.20 pg/mL, and 3.70 pg/mL. Standard concentrations in pmol/L are 1248.3, 499.3, 199.7, 79.8, 31.9 and 12.8.

- Pipette 18 mL of assay diluent into the disposable tube. Set aside for Step 5.

Step 4:

- Pipette 50 µL of standards and unknowns into appropriate wells. Standards and samples should be assayed in duplicate.
- Pipette 50 µL of assay diluent into 2 wells to serve as the zero.
- Pipette 50 µL of assay diluent into each NSB well.

Step 5: Dilute the enzyme conjugate by adding 7 µL of the conjugate to the 18 mL of assay diluent prepared in Step 2. 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 60 minutes at 500 rpm at room temperature.

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.

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**: *DO NOT mix at speeds over 600 rpms.* 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 (correction at 492 to 620 is desirable).

Calculations

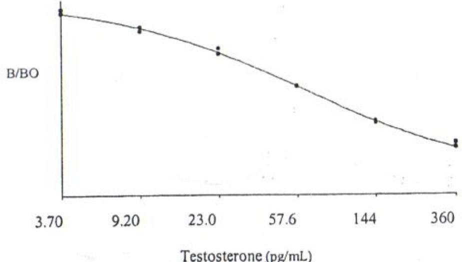
1. Compute the average optical density (OD) for all duplicate wells.
2. Subtract the average OD for the NSB wells from the average OD of the zero, standards, and unknowns/samples.
3. Calculate the percent bound (B/Bo) for each standard and unknown by dividing the average OD (B) by the average OD for the zero (Bo).
4. Determine the concentrations of the unknowns/samples by interpolation using software capable of logistics. We recommend using a 4-parameter sigmoid minus curve fit.

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	0.304	0.254	0.259	360
B1, B2	S2	0.426	0.376	0.384	144
C1, C2	S3	0.609	0.559	0.571	57.6
D1, D2	S4	0.793	0.743	0.759	23.0
E1, E2	S5	0.905	0.855	0.873	9.20
F1, F2	S6	0.997	0.947	0.967	3.70
G1, G2	Bo	1.029	0.979	NA	NA
H1, H2	NSB	0.05	NA	NA	NA

Example: Testosterone 4-Parameter Sigmoid Minus Curve Fit



Material Safety Data*

Hazardous Ingredients

Stop Solution is a 2 molar solution of sulfuric acid. This solution is caustic; use with care. We recommend the procedures listed below for all kit reagents.

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 contact with reagents.

Performance Characteristics

1. **Recovery** - Saliva samples containing different levels of endogenous testosterone were spiked with known quantities of testosterone and assayed. The average recovery was 105.0% (range 93.3% to 116.3%), 109.12% for males and 99.6% for females.
2. **Intra-assay precision** was determined from the mean of 8 replicates at high (197.3 pg/mL) and low (26.3 pg/mL) testosterone levels. The average intra-assay coefficient of variation was 3.3% and 6.7% for high and low levels.
3. **Inter-assay precision** was determined from the mean of averaged duplicates for 10 separate runs at high (200.7 pg/mL) and low (13.1 pg/mL) testosterone levels. The average inter-assay coefficient of variation was 5.1% for high and 9.6% for low testosterone levels.
4. **Linearity of dilution**- Saliva samples were diluted (range 1:2 to 1:16) with assay buffer and assayed in duplicate. The average recovery was 101.1% (range 88.2% to 120.2%), 102.86% for males and 98.68% for females.
5. **Sensitivity**- The lower limit of sensitivity was determined by interpolating the mean minus 2 SD for 10 sets of duplicates for the 0 pg/mL standard (4). The minimal concentration of testosterone that can be distinguished from 0 is <1.5 pg/mL.

6. **Correlation with serum** – The correlation between saliva and serum free and total testosterone was determined by assaying 32 matched samples (16 adult males and females). The saliva-serum free testosterone correlation was, $\underline{r}$ (30) = 0.93, $\underline{p} < 0.001$, and the saliva-serum total testosterone correlation was $\underline{r}$ (30) = 0.929, $\underline{p} < 0.001$. The serum-saliva correlations were stronger for males ($\underline{r}$ = 0.80 to 0.85) than for females ($\underline{r}$ = 0.38 to 0.48).
7. **Method Comparison** – The correlation between the Salimetrics EIA and a published serum RIA modified for use with saliva (2) was evaluated by assaying 32 common samples. For the combined sample, the EIA-RIA results were highly correlated, $\underline{r}$ (30) = 0.95, $\underline{p} < 0.001$. The EIA-RIA correlation was stronger for males ($\underline{r}$ = 0.88) than for females ($\underline{r}$ = 0.54).

†Salivary Testosterone Expected Ranges:

Adult males (mid day*)	70-220 pg/mL
Adult females (mid day*)	5-70 pg/mL
*AM values may be significantly higher	

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

References

1. Kirschbaum, C., Read, G.F., & Hellhammer, D.H. (1992). *Assessment of hormones and drugs in saliva in biobehavioral research.* Kirkland, WA: Hogefe & Huber.
2. Granger, D. A., Schwartz, E. B., Booth, A., & Arentz, M. (1999). Salivary testosterone determination in studies of child health and development. *Hormones and Behavior, 35*, 18-27.
3. Schwartz, E.B., Granger, D.A., Susman, E.J., Gunnar, M.R., & Laird, B. (1998). Assessing salivary cortisol in studies of child development. *Child Development, 69*, 1503-1513.
4. Chard, T. (1990). *An introduction to radioimmunoassay and related techniques.* Amsterdam: Elsevier.
5. Kivlighan, K. T., Granger, D. A., Schwartz, E. B., Nelson, V., & Curran, M. (2004). Quantifying blood leakage into the oral mucosa and its effects on the measurement of cortisol, dehydroepiandrosterone, and testosterone in saliva. *Hormones and Behavior, 46*, 39-46.
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7. Shirtcliff, E.A., Granger, D.A., Schwartz, E., & Curran, M.J. (2001). Use of salivary biomarkers in biobehavioral research: Cotton based sample collection methods can interfere with salivary immunoassay results. *Psychoneuroendocrinology, 26*, 165-173.

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."

Anexo 6 – Dados: Resultados do Teste de Nado

Tabela 3 – Distância do último parcial, tempo do ultimo parcial, distância percorrida nos 20 minutos, Lactato, Frequência gestual e Frequência Cardiaca (bpm).

Sujeitos	Dist.Últ.Parc	Tpo Últ Parc	Dist 20'	Lactato	Fg Média
01	1500	19:38,27	1528	2,6	26,367
02	1500	19:19,58	1552	3,1	30,900
03	1600	19:55,10	1607	3,2	24,316
04	1500	19:06,74	1570	3,0	26,938
05	1400	18:45,67	1492	1,8	32,879
06	1500	19:08,03	1568	3,9	34,531
07	1400	19:35,79	1429	2,8	28,950
08	1500	19:21,31	1550	2,6	26,240
09	1500	19:27,19	1542	2,8	26,731
10	1400	19:27,80	1439	2,6	28,929
11	1400	19:17,06	1452	2,8	25,521
12	1400	18:50,70	1486	4,1	31,600

Anexo 8 – Ficha de Registo de Placas - ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Anexo 11 – Tratamento Estatístico

Estatística Descritiva

	Mínimo	Máximo	Média	Desvio Padrão
Idade Décimal (anos)	15,33	18,64	17,03	,89
Anos de treino	6,00	9,00	7,33	,89
Massa corporal	55,20	79,60	66,45	7,17
Altura	164,50	191,60	177,11	7,17
Altura sentado	84,00	95,10	90,87	3,20
Envergadura	171,00	194,00	182,17	8,54
∑ Pregas	32,00	69,0	47,25	10,36

	Mínimo	Máximo	Média	Desvio Padrão
Velocidade Nado	1,191	1,339	1,26486	,047797
%VelMx	68,602	78,680	74,20638	3,056031
FC	135	169	156,75	11,194
Lactato	1,8	4,1	2,942	,6082

	Mínimo	Mínimo	Média	Desvio Padrão
Testo1	83,37	142,19	119,1864	18,81198
Testo2	76,93	190,58	129,9642	31,06603
Testo3	40,08	221,68	97,1196	50,14863
Testo4	58,49	147,66	90,6299	27,73428
Testo5	110,69	241,36	161,2997	41,31438
Testo6	46,19	152,63	108,4413	31,96822

	Mínimo	Máximo	Média	Desvio Padrão
Cortisol1	260,00	6450,00	1698,3333	1720,97136
Cortisol2	560,00	5200,00	1783,3333	1438,82107
Cortisol3	100,00	2010,00	879,1667	597,66908
Cortisol4	130,00	2000,00	531,6667	519,61233
Cortisol5	610,00	9100,00	3715,0000	2692,81029
Cortisol6	270,00	1820,00	874,1667	544,70106

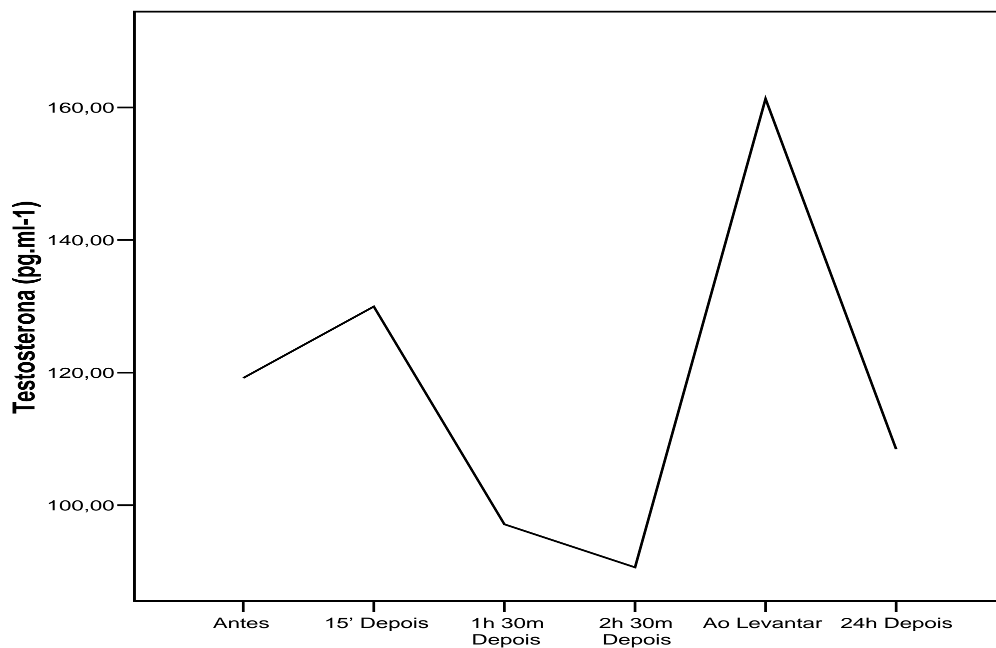
	Mínimo	**Máximo**	**Média**	**Desvio Padrão**
Racio1	,02	,50	,1456	,13290
Racio2	,03	,26	,1132	,07024
Racio3	,03	1,29	,2239	,34369
Racio4	,07	,61	,2775	,18509
Racio5	,02	,28	,0792	,07757
Racio6	,07	,31	,1666	,08993

Estatística Diferencial

Test Statistics(c)

	Testo2 - testo1	Testo3 - testo1	Testo4 - testo1	Testo5 - testo1	Testo6 - testo1
Z	-1,255(a)	-1,647(b)	-2,432(b)	-2,746(a)	-1,177(b)
Asymp. Sig. (2-tailed)	,209	,099	,015	,006	,239

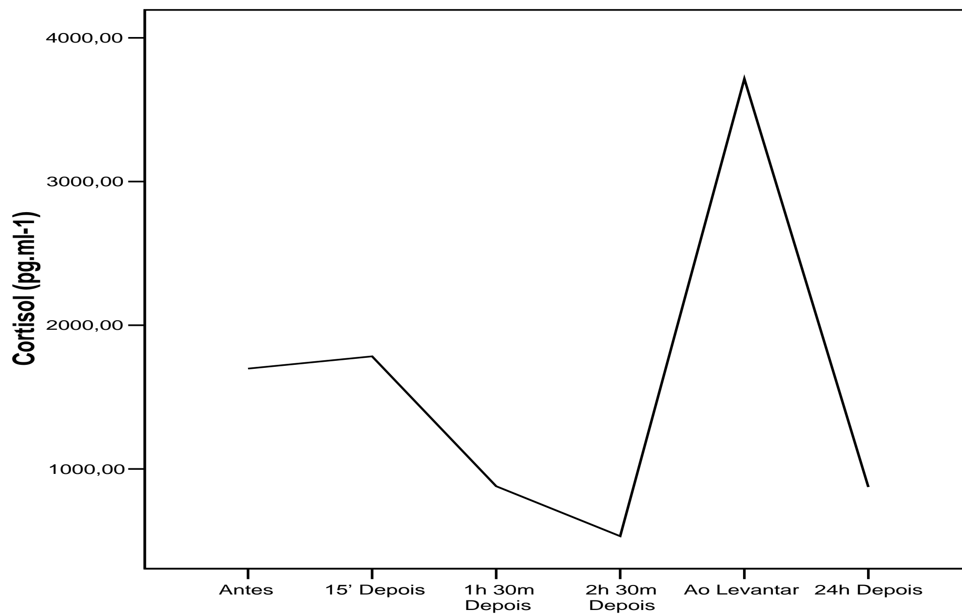
a Based on negative ranks.
b Based on positive ranks.
c Wilcoxon Signed Ranks Test



Test Statistics(c)

	Cortisol2 - Cortisol1	Cortisol3 - Cortisol1	Cortisol4 - Cortisol1	Cortisol5 - Cortisol1	Cortisol6 - Cortisol1
Z	-,157(a)	-1,883(b)	-2,040(b)	-1,883(a)	-1,726(b)
Asymp. Sig. (2-tailed)	,875	,060	,041	,060	,084

a Based on negative ranks.
b Based on positive ranks.
c Wilcoxon Signed Ranks Test



Test Statistics(c)

	Racio2 - Racio1	Racio3 - Racio1	Racio4 – Racio1	racio5 - Racio1	Racio6 - Racio1
Z	-,078(a)	-,392(b)	-1,569(b)	-2,118(a)	-1,255(b)
Asymp. Sig. (2-tailed)	,937	,695	,117	,034	,209

a Based on positive ranks.
b Based on negative ranks.
c Wilcoxon Signed Ranks Test