

SALIVARY ANDROSTENEDIONE ENZYME IMMUNOASSAY KIT

Catalog No. 1-2902/1-2912, 96-Well Kit

For Research Use

Intended Use

Salimetrics' salivary androstenedione kit is a competitive immunoassay specifically designed for the quantitative measurement of salivary androstenedione. It is not intended for use with serum/plasma. It is intended for research use with saliva. Please read the complete kit insert before performing this assay. 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

Androstenedione (4-androstene-3,17-dione) is produced in the adrenal gland and gonads. Androstenedione has weak intrinsic androgenic activity (estimated at less than 20% of testosterone), but it is a prohormone for potent androgens (testosterone) and estrogens. The biochemical evidence supporting the effect of androstenedione on elevation of circulating levels of testosterone and estrogens is strong, and site-of-action direct conversion of androstenedione to testosterone is well known (1). Secretion of androstenedione in women exceeds that of testosterone as significant extra-adrenal conversion of androstenedione to testosterone occurs in females. High levels of androstenedione may confer androgenic risk, especially in females, and estrogenic risks, especially in males. Children and adolescents are particularly vulnerable to the effects of androstenedione conversion to active sex steroids. These effects may disrupt normal sexual development, specifically virilization in girls associated with severe acne, excessive body hair, disruption of the menstrual cycle, and infertility. The conversion of androstenedione to estrogens can cause feminization of boys. In both boys and girls, the combined effects of excessive androgens and estrogens can induce premature puberty and significantly compromise adult stature by causing early closure of growth plates of long bones (2,3).

Measurement of serum androstenedione is used as a marker of androgen biosynthesis. High circulating androstenedione levels are indicated in virilizing congenital adrenal hyperplasia, polycystic ovarian syndrome, and other causes of hirsutism in women. Elevated androstenedione levels may also occur as a result of adrenal or ovarian tumors. The high serum-saliva correlation for androstenedione suggests that individual differences in serum androstenedione levels may be accurately estimated using saliva as a non-invasive alternative specimen (4,5).

The U.S. FDA has released high profile warnings to the public about serious health effects related to androstenedione administration (6). Androstenedione is an additive in many products generally advertised as dietary supplements that enhance athletic performance. Use of these supplements has the potential to cause extreme values in this assay. Also, it is noteworthy, that the effects of oral androstenedione administration on circulating levels of testosterone and estrogen do not account for all of the actions of these products. Target tissues of anabolic steroids contain abundant enzymes that convert circulating androstenedione to testosterone right at the site of action without necessarily affecting circulating testosterone levels.

To ensure the most accurate results, this salivary immunoassay is designed using a matrix that matches saliva. The level of androstenedione in saliva (pg/mL) is significantly lower than levels in the general circulation (ng/mL). The standard curve range is sensitive enough to capture individual differences in the androstenedione levels expected in saliva. The current protocol uses only 50 μ L of saliva per test. No separation or extractions are necessary.

Test Principle

A microtitre plate is coated with rabbit antibodies to androstenedione. Androstenedione in standards and unknowns compete with androstenedione linked to horseradish peroxidase for the antibody binding sites. After incubation, unbound components are washed away. Bound androstenedione 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 androstenedione peroxidase detected is inversely proportional to the amount of androstenedione present (7).

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. Androstenedione values from samples with a $\text{pH} \leq 4.0$ or ≥ 9.0 may be artificially inflated or lowered (8).

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 (9,10), 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 set of 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-Androstenedione Coated Plate:** A ready-to-use 96-well microtitre plate pre-coated with rabbit anti-androstenedione antibodies in a resealable foil pouch.
2. **Androstenedione Standard:** 1.0 mL of androstenedione, in a saliva-like matrix with a non-mercury preservative, at a concentration of 2430 pg/mL.
3. **Androstenedione Controls:** Two controls representing high and low levels of androstenedione in a saliva-like matrix with a non-mercury preservative. Each vial contains 0.5 mL. See vials for target ranges.
4. **Wash Buffer:** 100 mL of a 10X phosphate buffered solution containing detergents and a non-mercury preservative. Dilute only the amount needed for current day's use. Discard any leftover reagent. Dilute the wash buffer concentrate 10-fold with room temperature deionized water (100 mL of 10X wash buffer to 900 mL of deionized H₂O). (**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.*)
5. **Assay Diluent:** 63 mL of a phosphate buffered solution containing a pH indicator and a non-mercury preservative.
6. **Enzyme Conjugate:** 40 μ L of a solution of androstenedione labeled with horseradish peroxidase. Dilute prior to use with assay diluent.
7. **Tetramethylbenzidine (TMB):** 25 mL of a non-toxic ready-to-use solution.
8. **Stop Solution:** 12.5 mL of a 2-molar solution of sulfuric acid (USA customers only). Stop solution is provided in powdered form to customers outside the USA. Reconstitute the powdered stop solution with 12.5 mL of deionized water. Let sit for 10 minutes before using.
9. **Non-specific Binding Wells:** These wells do not contain anti-androstenedione 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 24 μ L, 50 μ L, and 150 μ L
- Precision multichannel pipette to deliver 50 μ L, 150 μ L, and 200 μ L
- 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

Due to the episodic secretion pattern of steroid hormones, we can only expect reproducible and reliable results in cases of multiple sampling. Therefore, we recommend taking 5 samples within at least a 2-hour period and pooling the samples before testing (11).

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 (12,13), 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.** Samples visibly contaminated with blood should be recollected. 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. 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	2430 Std	2430 Std	C-H	C-H								
B	810 Std	810 Std	C-L	C-L								
C	270 Std	270 Std	Unk-1	Unk-1								
D	90 Std	90 Std	Unk-2	Unk-2								
E	30 Std	30 Std	Unk-3	Unk-3								
F	10 Std	10 Std	Unk-4	Unk-4								
G	Zero	Zero	Unk-5	Unk-5								
H	NSB	NSB	Unk-6	Unk-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, controls or unknowns.

Step 3:

- Label five microcentrifuge tubes or other small tubes 2 through 6.
- Pipette 200 µL of assay diluent in tubes 2 through 6. Serially dilute the standard 3X by adding 100 µL of the 2430 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 2430 pg/mL, 810 pg/mL, 270 pg/mL, 90 pg/mL, 30 pg/mL, and 10 pg/mL. Standard concentrations in nmol/L are 8.484, 2.828, 0.943, 0.314, 0.105 and 0.035, respectively.
- Pipette 18 mL of assay diluent into the disposable tube (scale down proportionally if not using the entire plate). Set aside for Step 5.

Step 4:

- Pipette 50 µL of standards, controls and unknowns into appropriate wells. Standards, controls and unknowns 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 1:750 by adding 24 µL of the conjugate to the 18 mL of assay diluent prepared in Step 3. Immediately mix the diluted conjugate solution and add 150 µL to each well using a multichannel pipette. Cover plate with plate seal.

Step 6: Mix plate on a plate rotator for 5 minutes at 500 rpm and incubate at room temperature for an additional 115 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.

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 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. 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 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 (correction at 620 is desirable).

Calculations

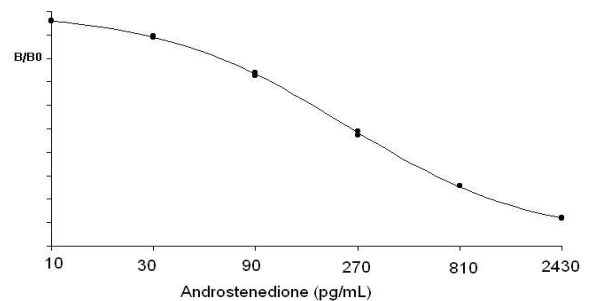
- Compute the average optical density (OD) for all duplicate wells.
- Subtract the average OD for the NSB wells from the average OD of the zero, standards, controls and unknowns (B).
- Calculate the percent bound (B/Bo) for each standard, control and unknown by dividing the average OD (B) by the average OD for the zero (Bo).
- 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.

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	Androstenedione (pg/mL)
A1, A2	S1	0.240	0.230	0.130	2430
B1, B2	S2	0.479	0.469	0.265	810
C1, C2	S3	0.891	0.881	0.498	270
D1, D2	S4	1.333	1.323	0.747	90
E1, E2	S5	1.620	1.610	0.910	30
F1, F2	S6	1.722	1.712	0.967	10
G1, G2	Bo	1.780	1.770	NA	NA
H1, H2	NSB	0.010	NA	NA	NA

Example: Androstenedione 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 be used only as a guide. Salimetrics shall not be liable for accidents or damage resulting from contact with reagents.

Performance Characteristics

A. Recovery:

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

Sample	Endogenous (pg/ml)	Added (pg/ml)	Expected (pg/ml)	Observed (pg/ml)	Recovery (%)
1	76.30	800	876.30	817.48	93.3
2	219.37	800	1019.37	1032.96	101.3
3	84.94	243	327.94	339.87	103.6
4	158.05	243	401.05	430.85	107.43
5	84.94	9	93.94	98.56	104.9
6	158.05	9	167.05	164.49	98.5

B. Precision:

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

Sample	N	Mean (pg/ml)	Standard Deviation (pg/ml)	Coefficient of Variation (%)
H	12	1065.61	16.33	1.5
L	12	45.28	3.39	7.5

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

Sample	N	Mean (pg/ml)	Standard Deviation (pg/ml)	Coefficient of Variation (%)
H	12	1061.19	40.38	3.8
L	12	40.54	3.44	8.5

C. Sensitivity:

The lower limit of sensitivity was determined by interpolating the mean minus 2 SD's for 10 sets of duplicates at 0 pg/ml standard. The minimal concentration of androstenedione that can be distinguished from 0 is 5.0 pg/ml.

D. Correlation with serum:

The correlation between saliva and total serum androstenedione was determined by assaying 35 matched samples (17 adult males and 18 females). The correlation between serum and saliva androstenedione is highly significant, $r(33) = 0.77$, $p < 0.001$.

E. Linearity of Dilution:

Two saliva samples were serially diluted with assay diluent and assayed.

Sample	Dilution Factor	Expected (pg/ml)	Observed (pg/ml)	Recovery (%)
1			362.12	
	1:2	181.06	192.84	106.5
	1:4	90.53	100.14	110.6
	1:8	45.27	38.48	85.0
	1:16	22.63	23.90	105.6
2			1193.01	
	1:2	596.51	540.19	90.6
	1:4	298.25	302.73	101.5
	1:8	149.13	151.68	101.7
	1:16	74.56	70.06	94.0

F. Specificity

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

Compound	Spiked Concentration (ng/mL)	% Cross-reactivity
Testosterone	1000	0.250
DHEA	1000	0.243
DHEA-S	1000	0.007
Progesterone	1000	0.022
17- α Hydroxy-progesterone	1000	ND
Estradiol	10	0.541
Estrone	1000	0.006
Estriol	1000	ND
Aldosterone	1000	ND
Cortisol	1000	ND
Cortisone	1000	ND
11-Deoxycortisol	1000	ND
21-Deoxycortisol	1000	0.033
Dexamethasone	1000	ND
Triamcinolone	1000	ND
Corticosterone	1000	ND
Prednisolone	1000	ND
Prednisone	1000	ND
Transferrin	1000	ND

ND = None detected (< 0.004)

G. Expected Ranges in Males

Age (yrs)	N	AM Mean (pg/ml)	AM Std Dev (pg/ml)	PM Mean (pg/ml)	PM Std Dev (pg/ml)
6-8	5	53.53	22.74	29.42	12.34
9-10	10	76.76	38.88	68.32	28.34
11-12	9	134.52	52.81	84.93	24.12
13-14	9	147.52	41.32	86.11	33.31
15-16	10	156.76	40.26	117.77	48.52
17-18	5	208.29	69.74	158.02	41.51
Total	48	128.81	62.56	90.36	46.97
Adult	15	194.97	74.22	118.78	44.42

H. Expected Ranges in Females

Age (yrs)	N	AM Mean (pg/ml)	AM Std Dev (pg/ml)	PM Mean (pg/ml)	PM Std Dev (pg/ml)
6-8	6	59.78	18.63	66.64	47.02
9-10	10	93.52	45.41	66.93	29.24
11-12	9	162.98	70.10	149.64	67.50
13-14	9	211.15	105.48	156.51	72.56
15-16	8	233.16	102.97	171.94	43.59
17-18	5	272.48	80.98	155.74	32.61
Total	47	167.85	102.19	125.94	66.84
Adult	13	221.05	94.99	161.21	59.69

References

- Dorfman, R. I., Shipley, R. A. (1956). *Androgens*. New York: John Wiley and Sons.
- King, D.S., Sharp, R.L., Vukovich, M.D., Brown, G.A., Reifenrath, T.A., Uhl, N.L., & Parsons, K.A. (1999). Effect of oral androstenedione on serum testosterone and adaptations to resistance training in young men: a randomized controlled trial. *JAMA*, 281(21), 2020-8.
- Leder, B.Z., Longcope, C., Catlin, D.H., Ahrens, B., Schoenfeld, D.A., & Finkelstein, J.S. (2000). Oral androstenedione administration and serum testosterone concentrations in young men. *JAMA*, 283(6), 779-82.
- Leder, B.Z., Leblanc, K.M., Longcope, C., Lee, H., Catlin, D.H., & Finkelstein, J.S. (2002). Effects of oral androstenedione administration on serum testosterone and estradiol levels in postmenopausal women. *Journal of Clinical Endocrinology & Metabolism*, 87(12), 5449-54.
- Kicman, A.T., Bassindale, T., Cowan, D.A., Dale, S., Hutt, A.J., & Leeds, A.R. (2003). Effect of androstenedione ingestion on plasma testosterone in young women; a dietary supplement with potential health risks. *Clinical Chemistry*, 49 (1), 167-9.
- U.S. Food and Drug Administration. (2004). *Health effects of androstenedione*. (FDA White Paper). U. S. Department of Health and Human Services.
- Chard, T. (1990). *An introduction to radioimmunoassay and related techniques*. Amsterdam: Elsevier.
- 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.
- 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.
- Schwartz, E., & Granger, D. A. (2004). Transferrin enzyme immunoassay for quantitative monitoring of blood contamination in saliva. *Clinical Chemistry*, 50, 654-656.
- West, C.D., Mahajan, D.K., Chavre, V.J., Nabors, C.J. (1973). Simultaneous measurement of multiple plasma steroids by radioimmunoassay demonstrating episodic secretion. *Journal of Clinical Endocrinology & Metabolism*, 36 No.6, 1230 – 1236.
- Kirschbaum, C., Read, G.F., & Hellhammer, D.H. (1992). *Assessment of hormones and drugs in saliva in biobehavioral research*. Kirkland, WA: Hogefe & Huber.
- 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."