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SALIVARY ANDROSTENEDIONE ENZYME IMMUNOASSAY KIT

Catalog No. 1-2902, (Single) 96-Well Kit;
1-2902-5, (5-Pack) 480 Wells

For Research Use Only

Intended Use

The Salimetrics™ salivary androstenedione kit is a competitive immunoassay specifically designed and validated for the quantitative measurement of salivary androstenedione. It is not intended for diagnostic use. It is intended only for research use in humans and some animals. 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 congential 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 androstendione 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 µL 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 competes 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 pH $\leq$ 4.0 or $\geq$ 9.0 may be artificially inflated or lowered (8).

Precautions

1. Failure to follow kit procedure and recommendations for saliva collection and sample handling may result in false values.
2. Liquid stop solution is a 2-molar solution of sulfuric acid. This solution is caustic; use with care.
3. This kit uses break-apart microtitre strips. Unused wells must be stored at 2 - 8°C in the sealed foil pouch with desiccant and used in the frame provided.
4. Do not mix components from different lots of kits.
5. 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.
6. See 'Material Safety Data' at the end of procedure.
7. 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.
8. Routine calibration of pipettes is critical for the best possible assay performance.
9. Pipetting of samples and reagents must be done as quickly as possible (without interruption) across the plate.
10. When running multiple plates, or multiple sets of strips, a standard curve should be run with each individual plate and/or set of strips.
11. The temperature of the laboratory may affect assays. Salimetrics' kits have been validated at 68 - 74ºF (20 - 23.3ºC). Higher or lower temperatures will cause an increase or decrease in OD values, respectively. Salimetrics cannot guarantee test results outside of this temperature range.
12. The quantity of reagent provided with this kit 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.
13. Avoid microbial contamination of opened reagents. Salimetrics recommends using opened reagents within one month.

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.
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 H_20). (**Note:** *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.)*
5. **Assay Diluent:** 60 mL of a phosphate buffered solution containing a pH indicator and a non-mercury preservative.
6. **Enzyme Conjugate:** 40 µL 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.
9. **Non-specific Binding Wells (NSB):** 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 as blanks (optional) where needed.

Materials Needed But Not Supplied

- Precision pipette to deliver 24 µL, 50 µL, and 150 µL
- Precision multichannel pipette to deliver 50 µL, 150 µL, and 200 µL
- Vortex
- Plate rotator with 0.08-0.17" orbit (assay sensitivity may be affected if a rotator is not used)
- Plate reader with a 450 nm filter
- Computer software for data reduction
- Deionized water
- Reagent reservoirs
- One 20 mL disposable tube
- Five small disposable tubes
- Pipette tips
- 25 mL serological pipette

Specimen Collection

Due to the episodic secretion pattern of steroid hormones, we can expect reproducible and reliable results only in cases of multiple sampling. Therefore, we recommend taking a minimum of 3 samples within at least a 2-hour period and pooling the samples before testing (11,12).

The preferred method of collecting whole saliva is by unstimulated passive drool. Collection protocols are available on request. **Do not use Salivettes, the SalivaBio Oral Swab (SOS), Sorbettes, cotton, or polyester materials to collect samples**. False readings will result (13,14). Do **not** add sodium azide to saliva samples as a preservative. Samples visibly contaminated with blood should be recollected. 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. Record the time and date of specimen collection. 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 -20ºC within 4 hours of collection. (Samples may be stored at -20ºC or lower for long term storage.)

Freezing saliva samples will 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-thaw cycles. However, if samples have been refrozen, centrifuge again prior to assaying. Samples should be at room temperature before adding to assay plate. 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. **Note***: It is important to keep the zip-lock pouch with the plate strips closed until warmed to room temperature as humidity may have an effect on the coated wells. Mix all reagents before use.*

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. Break off the bottom wells in each strip. 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 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:

- 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. (Scale down proportionally if not using the entire plate.) Immediately mix the diluted conjugate solution and add 150 µL to each well using a multichannel pipette. 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 630 is desirable.)

Calculations

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

Quality Control

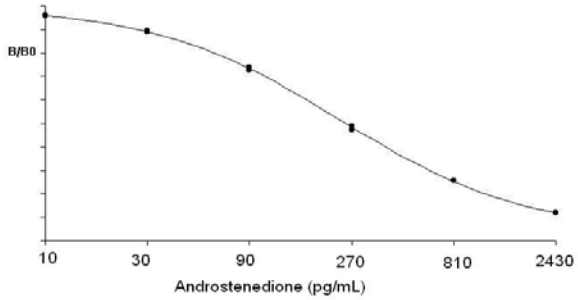
The Salimetrics' high and low salivary Androstenedione controls should be run with each assay. The control ranges established at Salimetrics are to be used as a guide. Each laboratory should establish its own range. Variations between laboratories may be caused by differences in techniques and instrumentation.

Typical Results

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

Well	Sample	Average OD	B	B/Bo	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

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

Handling

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

Emergency Exposure Measures

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

*The above information is believed to be accurate but is not all-inclusive. This information should only be used as a guide. Salimetrics shall not be liable for accidents or damage resulting from 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. 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 androstenedione that can be distinguished from 0 is 5.0 pg/ml.

C. 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

D. 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

E. 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
Dihydrotestosterone	1000	0.069
19-Nortestosterone	1000	0.020
11-Hydroxytestosterone	1000	ND
DHEA	1000	0.243
DHEA-S	1000	0.007
Dianabol	1000	0.022
Progesterone	1000	0.022
17 α-Hydroxyprogesterone	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
Triamincinolone	1000	ND
Corticosterone	1000	ND
Prednisolone	1000	ND
Prednisone	1000	ND
Transferrin	1000	ND

ND = None detected (< 0.004)

F. 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

G. 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

H. 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 androstendione is highly significant, $r(33) = 0.77$, $p < 0.001$.

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Seller's Limited Warranty

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

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