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DCM027-9
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ANDROSTENEDIONE SALIVA ELISA

for routine analysis

Direct immunoenzymatic determination of Androstenedione in saliva

IVD

LOT
See external label

2°C 8°C

Σ = 96 tests

REF DKO027

INTENDED USE

Eagle Biosciences Androstenedione Saliva ELISA Assay Kit is a competitive immunoenzymatic colorimetric method for quantitative determination of Androstenedione concentration in saliva. Androstenedione Saliva ELISA Assay Kit is intended for research use only.

1. CLINICAL SIGNIFICANCE

Androstenedione (also known as Δ4-androstenedione) is a steroid hormone produced in the adrenal glands and the gonads as an intermediate step in the biochemical pathway that produces the androgen testosterone and the estrogens estrone and estradiol. It is the common precursor of male and female sex hormones. Some androstenedione is also secreted into the plasma, and may be converted in peripheral tissues to testosterone and estrogens.

Androstenedione has relatively weak androgenic activity, estimated at ~ 20% of testosterone. Secretion and production rates also exceed those of testosterone in women in whom significant extra-adrenal conversion of androstenedione to testosterone occurs.

In premenopausal women the adrenal glands and ovaries each produce about half of the total androstendione (about 3 mg/day). After menopause androstenedione production is about halved, primarily due to the reduction of steroid secreted by the ovary. Nevertheless, androstenedione is the principal steroid produced by the postmenopausal ovary.

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.

2. PRINCIPLE

In Androstenedione Saliva ELISA Assay Kit, the Androstenedione (antigen) in the sample competes with the antigenic Androstenedione conjugated with horseradish peroxidase (HRP) for binding to the limited number of antibodies anti Androstenedione coated on the microplate (solid phase).

After incubation, the bound/free separation is performed by a simple solid-phase washing. Then, the enzyme HRP in the bound-fraction reacts with the Substrate (H_2O_2) and the TMB Substrate and develops a blu color that changes into yellow when the Stop Solution (H_2SO_4) is added.

The color intensity is inversely proportional to the Androstenedione concentration in the sample. Androstenedione concentration in the sample is calculated through a calibration curve.

3. REAGENTS, MATERIALS AND INSTRUMENTATION

3.1. Reagent and material supplied in the kit

1. Androstenedione Calibrators (5 vials, 1 mL each)

CAL0	REF **DCE002/2706-0**
CAL1	REF **DCE002/2707-0**
CAL2	REF **DCE002/2708-0**
CAL3	REF **DCE002/2709-0**
CAL4	REF **DCE002/2710-0**

2. Incubation Buffer (1 vial, 30 mL)
Phosphate buffer pH 7.5, BSA 1 g/L
REF **DCE001-0**

3. Conjugate (1 vial, 1 mL)
Androstenedione conjugated with horseradish peroxidase (HRP) REF **DCE002/2702-0**

4. Coated Microplate (1 breakable microplate)
Anti androstenedione antibody adsorbed on microplate REF **DCE002/2703-0**

5. TMB Substrate (1 vial, 15 mL)
H_2O_2-TMB 0.26 g/L *(avoid any skin contact)*
REF **DCE004-0**

6. Stop Solution (1 vial, 15 mL)
Sulphuric acid 0.15 mol/L *(avoid any skin contact)*
REF **DCE005-0**

7. 50X Conc. Wash Solution (1 vial, 20 mL)
NaCl 45 g/L; Tween-20 55 g/L REF **DCE006-0**

3.2. Reagents necessary not supplied

Distilled water

3.3. Auxiliary materials and instrumentation

Automatic dispenser
Microplates reader (450 nm)
Saliva Collection Device REF **DKO063**

Note

Store all reagents at 2-8°C in the dark.
Open the bag of reagent 4 (Coated Microplate) only when it is at room temperature and close it immediately after use; once opened, the microplate is stable until the expiry date of kit. Do not remove the adhesive sheets on the unused strips

4. WARNINGS

- This Androstenedione Saliva ELISA Assay Kit is intended for reserach use by professional persons only. Not for internal or external use in Humans or Animals.
- Use appropriate personal protective equipment while working with the reagents provided.
- Follow Good Laboratory Practice (GLP) for handling blood products.
- Some reagents of the Androstenedione Saliva ELISA Assay Kit contain small amounts of Proclin 300^{R} as preservatives. Avoid the contact with skin or mucosa.
- The TMB Substrate contains an irritant, which may be harmful if inhaled, ingested or absorbed through the skin. To prevent injury, avoid inhalation, ingestion or contact with skin and eyes.
- The Stop Solution consists of a diluted sulphuric acid solution. Sulphuric acid is poisonous and corrosive and can be toxic if ingested. To prevent chemical burns, avoid contact with skin and eyes.
- Avoid the exposure of reagent TMB/H_2O_2 to directed sunlight, metals or oxidants. Do not freeze the solution.
- This Androstenedione Saliva ELISA Assay Kit allows the determination of Androstenedione from 5 pg/mL to 1000 pg/mL.
- The clinical significance of Androstenedione determination can be invalidated if the patient was treated with cortisone or natural or synthetic steroids.

5. PRECAUTIONS

- Please adhere strictly to the sequence of pipetting steps provided in this protocol. The performance data represented here were obtained using specific reagents listed in this Instruction For Use.
- All reagents of the Androstenedione Saliva ELISA Assay Kit should be stored refrigerated at 2-8°C in their original container. Any exceptions are clearly indicated. The reagents are stable until the expiry date when stored and handled as indicated.
- Allow all Androstenedione Saliva ELISA Assay Kit components and specimens to reach room temperature (22-28°C) and mix well prior to use.
- Do not interchange Androstenedione Saliva ELISA Assay Kit components from different lots. The expiry date printed on box and vials labels must be observed. Do not use any kit component beyond their expiry date.
- If you use automated equipment, the user has the responsibility to make sure that the kit has been appropriately tested.
- The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or increase the background.
- It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond ten minutes to avoid assay drift. If more than 10 minutes are needed, follow the same order of dispensation. If more than one plate is used, it is recommended to repeat the dose response curve in each plate
- Addition of the TMB Substrate solution initiates a kinetic reaction, which is terminated by the addition of the Stop Solution. Therefore, the TMB Substrate and the Stop Solution should be added in the same sequence to eliminate any time deviation during the reaction.
- Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera.
- Maximum precision is required for reconstitution and dispensation of the reagents.
- Samples microbiologically contaminated, highly lipemeic or haemolysed should not be used in the assay.
- Plate readers measure vertically. Do not touch the bottom of the wells.

6. PROCEDURE

6.1. Preparation of the Calibrators (C_0…C_4)

Before use, mix for 5 minutes with a rotating mixer.
The Calibrators are ready to use and have the following concentration of Androstenedione:

	C_0	C_1	C_2	C_3	C_4
pg/mL	0	20	100	400	1000

For samples with Androstenedione concentration greater than 1000 pg/mL dilute the sample (1:2) with C_0.
Once opened, the Calibrators are stable 6 months at 2-8°C.
For SI UNITS: pg/mL x 3,487 = pmol/L

6.2. Preparation of Diluted Conjugate

Prepare immediately before use.
Add 10 µL of Conjugate (reagent 3) to 1.0 mL of Incubation Buffer (reagent 2). Mix gently. Stable 3 hours at room temperature (22÷28°C).

6.3. Preparation of Wash Solution

Dilute the contents of each vial of the buffered wash solution concentrate (50x) with distilled water to a final volume of 1000 mL prior to use. For smaller volumes respect the 1:50 dilution ratio. The diluted wash solution is stable for 30 days at 2÷8°C.

6.4. Preparation of the Sample

This kit allows the determination of Androstenedione concentration in saliva samples.
It is recommended to collect saliva samples with a centrifuge glass tube and a plastic straw, with the Diametra *Saliva Collection Device* or with the "Salivette" (Sarstedt, Ref. 511534500). Other commercially available sample collector devices have not been tested.

6.4.1. Method and Limitations

Collect saliva samples at the times indicated.
If no specific instructions have been given, saliva samples may be collected at any time, paying attention to the following indications:

a) If saliva collection is carried out in the morning ensure that this is carried out prior to brushing teeth
b) During the day allow 1 hour after a meal, oral intake of pharmaceutical drugs or tooth cleaning.
c) It is very important that a good clear sample is received – i.e. no contamination with food, lipstick, blood (bleeding gums) or other extraneous materials.

6.4.2. Saliva Processing Instructions with *Saliva Collection Device Diametra*

1) Let the saliva flow down trought the straw into the centrifuge glass tube.
2) Centrifuge the sample for 15 minutes at 3000 rpm
3) Store at – 20°C for at least 1 hour
4) Centrifuge again for 15 minutes at 3000 rpm
5) The saliva sample is now ready to be tested.
6) Store the sample at 2÷8°C for one week or at – 20°C for longer time.

6.4.3. Saliva Processing Instructions with *Salivette Sardstedt*

1) Remove the swab from the suspended insert of the Salivette
2) Gently chewing the swab for 1 minute produces a sufficient quantity of saliva.
3) Replace the swab into the Salivette and firmly close the tube using the stopper.
4) Centrifuge the Salivette for 2 minutes at 1000g (rcf) for saliva generation.
5) Remove the insert complete with the swab from the centrifuge vessel and discard. The clear saliva is now ready for analysis (at least 1 mL of saliva should be recovered with this method).

6.5. Procedure

- **Allow all reagents to reach room temperature (22-28°C).**
- Unused coated microwell strips should be released securely in the foil pouch containing desiccant and stored at 2-8°C.
- To avoid potential microbial and/or chemical contamination, unused reagents should never be transferred into the original vials.
- As it is necessary to perform the determination in duplicate in order to improve accuracy of the test results, prepare two wells for each point of the calibration curve (C_0-C_4), two for each Control, two for each sample, one for Blank.

Reagent	Calibrator	Samples	Blank
Calibrator C_0-C_4	**50 µL**		
Samples		**50 µL**	
Diluted Conjugate	**150 µL**	**150 µL**	
Incubate at +37°C for 1 hour. Remove the contents from each well; wash the wells 3 times with 300 µL of diluted Wash Solution.			
TMB Substrate	**100 µL**	**100 µL**	**100 µL**
Incubate at room temperature (22-28°C) for 15 minutes in the dark.			
Stop Solution	**100 µL**	**100 µL**	**100 µL**
Shake the microplate gently. Read the absorbance (E) at 450 nm against Blank within 5 minutes.			

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7. QUALITY CONTROL

Each laboratory should assay controls at normal, high and low levels range of Androstenedione for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. The individual laboratory should set acceptable assay performance limits. Other parameters that should be monitored include the 80, 50 and 20% intercepts of the calibration curve for run-to-run reproducibility. In addition, maximum absorbance should be consistent with past experience. Significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

8. RESULTS

8.1. Mean Absorbance

Calculate the mean of the absorbance (Em) for each point of the calibration curve (C_0-C_4) and of each sample.

8.2. Calibration curve

Plot the mean value of absorbance (Em) of the Calibrators (C_0-C_4) against concentration. Draw the best-fit curve through the plotted points. (es: Four Parameter Logistic).

8.3. Calculation of Results

Interpolate the values of the samples on the calibration curve to obtain the corresponding values of the concentrations expressed in pg/mL.

9. REFERENCE VALUES

As the values of salivary Androstenedione have a cicardian pattern we suggest to collect the samples at the same hour (8 A.M.):
The following values can be used as preliminary guideline until each laboratory established its own normal range.

		pg/mL
WOMEN:	Normal	20 – 160
	P.C.O.- Hirsute	120 – 300
MEN:		20 - 150

Please pay attention to the fact that the determination of a range of expected values for a "normal" population in a given method is dependent on many factors, such as specificity and sensitivity of the method used and type of population under investigation. Therefore each laboratory should consider the range given by the Manufacurer as a general indication and produce their own range of expected values based on the indigenous population where the laboratory works.

10. PERFORMANCE AND CHARACTERISTICS

10.1. Precision

10.1.1. Intra Assay Variation

Within run variation was determined by replicate (16x) the measurements of two different saliva controls in one assay. The within assay variability is ≤ 8.5%.

10.1.2. Inter Assay Variation

Between run variation was determined by replicate (10x) the measurements of two different saliva controls with different lots of kit. The between assay variability is ≤ 11%.

10.2. Accuracy

The recovery of 50 – 200 – 500 of Androstenedione added to sample gave an average value (±SD) of 102.60% ± 13.23% with reference to the original concentrations.

10.3. Sensitivity

The lowest detectable concentration of Androstenedione that can be distinguished from the Calibrator 0 is 5 pg/mL at the 95% confidence limit.

10.4. Specificity

The cross reaction of the antibody calculated at 50% according to Abraham are shown in the table:

Androstenedione Saliva	100 %
Testosterone	1.2 %
Epitestosterone	0.2 %
5α-dihydrotestosterone	0.1 %
DHEA	0.1 %
Progesterone	$1x10^{-3}$ %
Estrone	$1x10^{-3}$ %
Cortisol	$1x10^{-3}$ %

10.5. Correlation

Diametra Androstenedione saliva ELISA kit was compared to another commercially available Androstenedione saliva assay. 38 saliva samples were analysed according in both test systems.
The linear regression curve was calculated:
$y = 0.46x + 5.51$
$r^2 = 0.983$
y = Androstenedione saliva Diametra Elisa kit
x = Salivary Androstenedione Salimetrics Elisa kit

11. WASTE MANAGEMENT

Reagents must be disposed off in accordance with local regulations.

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ERROR POSSIBLE CAUSES / SUGGESTIONS

No colorimetric reaction

- no conjugate pipetted reaction after addition
- contamination of conjugates and/or of substrate
- errors in performing the assay procedure (e.g. accidental pipetting of reagents in a wrong sequence or from the wrong vial, etc.)

Too low reaction (too low ODs)

- incorrect conjugate (e.g. not from original kit)
- incubation time too short, incubation temperature too low

Too high reaction (too high ODs)

- incorrect conjugate (e.g. not from original kit)
- incubation time too long, incubation temperature too high
- water quality for wash buffer insufficient (low grade of deionization)
- insufficient washing (conjugates not properly removed)

Unexplainable outliers

- contamination of pipettes, tips or containers

insufficient washing (conjugates not properly removed) too high within-run

- reagents and/or strips not pre-warmed to CV% Room Temperature prior to use
- plate washer is not washing correctly (suggestion: clean washer head)

too high between-run - incubation conditions not constant (time, CV % temperature)

- controls and samples not dispensed at the same time (with the same intervals) (check pipetting order)
- person-related variation