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DCM027-12
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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

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INTENDED USE

Competitive immunoenzymatic colorimetric method for quantitative determination of Androstenedione concentration in saliva.
Androstenedione Saliva ELISA kit is intended for laboratory use only.

1. CLINICAL SIGNIFICANCE

Androstenedione (also known as 4-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

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 colour 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. 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. Controls (2 vials, 1 mL each)

Control A	REF DCE045/2703A-0
Control B	REF DCE045/2703B-0

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

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

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

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

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

8. 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, 620-630 nm)
Saliva Collection Device REF DKO063

Note

Store all reagents at 2÷8°C in the dark.

Open the bag of reagent 5 (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 kit is intended for in vitro 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 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 method 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 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 kit components and specimens to reach room temperature (22-28°C) and mix well prior to use.
- Do not interchange 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. To improve the performance of the kit on automatic systems is recommended to increase the number of washes.
- 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 L 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 and Controls

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 Dia.Metra *Saliva Collection Device*. Other commercially available sample collector devices have not been tested.
The controls are ready to use.

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

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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 Dia.Metra*

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.

6.5. Procedure

- **Allow all reagents to reach room temperature (22-28°C).** At the end of the assay, store immediately the reagents at 2-8°C: avoid long exposure to room temperature.
- 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	Sample / Control	Blank
Calibrator C_0-C_4	**50 µL**		
Sample / Control		**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. **Important note**: during each washing step, gently shake the plate for 5 seconds and remove excess solution by tapping the inverted plate on an absorbent paper towel. **Automatic washer**: if you use automated equipment, wash the wells at least 5 times.			
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 a reference wavelength of 620-630 nm or 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

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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

Dia.Metra 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 Dia.Metra 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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DiaMetra	Packaging Information Sheet	Mod. PIS000-1

IVD	DE ES FR GB IT PT	In vitro Diagnostikum Producto sanitario para diagnóstico In vitro Dispositif medical de diagnostic in vitro In vitro Diagnostic Medical Device Dispositivo medico-diagnostico in vitro Dispositivos medicos de diagnostico in vitro		DE ES FR GB IT PT	Hergestellt von Elaborado por Fabriqué par Manufacturer Produttore Produzido por
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SUGGERIMENTI PER LA RISOLUZIONE DEI PROBLEMI/TROUBLESHOOTING

ERRORE CAUSE POSSIBILI/ SUGGERIMENTI

Nessuna reazione colorimetrica del saggio

- mancata dispensazione del coniugato
- contaminazione del coniugato e/o del Substrato
- errori nell'esecuzione del saggio (es. Dispensazione accidentale dei reagenti in sequenza errata o provenienti da flaconi sbagliati, etc.)

Reazione troppo blanda (OD troppo basse)

- coniugato non idoneo (es. non proveniente dal kit originale)
- tempo di incubazione troppo breve, temperatura di incubazione troppa bassa

Reazione troppo intensa (OD troppo alte)

- coniugato non idoneo (es. non proveniente dal kit originale)
- tempo di incubazione troppo lungo, temperatura di incubazione troppa alta
- qualità scadente dell'acqua usata per la soluzione di lavaggio (basso grado di deionizzazione,)
- lavaggi insufficienti (coniugato non completamente rimosso)

Valori inspiegabilmente fuori scala

- contaminazione di pipette, puntali o contenitori- lavaggi insufficienti (coniugato non completamente rimosso)

CV% intrasaggio elevato

- reagenti e/o strip non portate a temperatura ambiente prima dell'uso
- il lavatore per micropiastre non lava correttamente (suggerimento: pulire la testa del lavatore)

CV% intersaggio elevato

- condizioni di incubazione non costanti (tempo o temperatura)
- controlli e campioni non dispensati allo stesso tempo (con gli stessi intervalli) (controllare la sequenza di dispensazione)
- variabilità intrinseca degli operatori

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

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ERROR / POSIBLES CAUSAS / SUGERENCIAS

No se produce ninguna reacción colorimétrica del ensayo

- no se ha dispensado el conjugado
- contaminación del conjugado y/o del substrato
- errores en la ejecución del ensayo (p. ej., dispensación accidental de los reactivos en orden incorrecto o procedentes de frascos equivocados, etc.)

Reacción escasa (DO demasiado bajas)

- conjugado no idóneo (p. ej., no procedente del kit original)
- tiempo de incubación demasiado corto, temperatura de incubación demasiado baja

Reacción demasiado intensa (DO demasiado altas)

- conjugado no idóneo (p. ej., no procedente del kit original)
- tiempo de incubación demasiado largo, temperatura de incubación demasiado alta
- calidad escasa del agua usada para la solución de lavado (bajo grado de desionización)
- lavados insuficientes (el conjugado no se ha retirado completamente)

Valores inexplicablemente fuera de escala

- contaminación de pipetas, puntas o contenedores- lavados insuficientes (el conjugado no se ha retirado completamente)

CV% intraensayo elevado

- los reactivos y/o tiras no se encontraban a temperatura ambiente antes del uso
- el lavador de microplacas no funciona correctamente (sugerencia: limpiar el cabezal del lavador)

CV% interensayo elevado

- condiciones de incubación no constantes (tiempo o temperatura)
- controles y muestras no dispensados al mismo tiempo (con los mismos intervalos) (controlar la secuencia de dispensación)
- variación en función de los operadores

ERREUR CAUSES POSSIBLES / SUGGESTIONS

Aucune réaction colorimétrique de l'essai

- non distribution du conjugué
- contamination du conjugué et/ou du substrat
- erreurs dans l'exécution du dosage (par ex., distribution accidentelle des réactifs dans le mauvais ordre ou en provenance des mauvais flacons, etc.)

Réaction trop faible (DO trop basse)

- conjugué non approprié (par ex., ne provenant pas du coffret original)
- temps d'incubation trop court, température d'incubation trop basse

Réaction trop intense (DO trop élevée)

- conjugué non approprié (par ex., ne provenant pas du coffret original)
- temps d'incubation trop long, température d'incubation trop élevée
- mauvaise qualité de l'eau utilisée pour la solution de lavage (bas degré de déionisation)
- lavages insuffisants (conjugué non entièrement éliminé)

Valeurs inexplicablement hors plage

- contamination des pipettes, embouts ou récipients - lavages insuffisants (conjugué non entièrement éliminé)

CV% intra-essai élevé

- les réactifs et/ou les bandes n'ont pas atteint la température ambiante avant usage
- le laveur de microplaques ne lave pas correctement (suggestion : nettoyer la tête du laveur)

CV% inter-essai élevé

- conditions d'incubation non constantes (temps ou température)
- contrôles et échantillons non distribués en même temps (avec les mêmes intervalles) (contrôler l'ordre de distribution)
- variabilité intrinsèque des opérateurs

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