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User's Manual

Androstenedione free in Saliva ELISA

IVD

CE

REF **DESLV4780**

Σ **96**

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DMC
Updated 160811

Please use only the valid version of the package insert provided with the kit.
Verwenden Sie nur die jeweils gültige, im Testkit enthaltene, Arbeitsanleitung.

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1 INTENDED USE

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

1.1 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 produces about half of the total androstendione (about 3 mg/day). After menopause the production of androstenedione decreases by 50%. This is mainly due to the reduction of the 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

Androstenedione (antigen) in the sample competes with the antigenic Androstenedione conjugated with horseradish peroxidase (HRP) for binding onto 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 blue color that changes into yellow when the Stop Solution (H_2SO_4) is added. The colour intensity is inversely proportional to the Androstenedione concentration of in the sample. Androstenedione concentration in the sample is calculated through a standard curve.

3 REAGENT, MATERIAL AND INSTRUMENTATION

3.1 Reagent and material supplied in the kit

1. **CAL | 0-4 Androstenedione Standards** S0 – S4 (5 vials, 1 mL each)
2. **CONTROL | 1-2 Controls** (2 vials, 1 mL each)
3. **INC | BUF Incubation Buffer** (1 vial, 30 mL)
   Phosphate buffer pH 7.5 BSA 1 g/L,
4. **ENZ | CONJ | 101x Enzyme Conjugate** (1 vial, 1.0 mL)
   Androstenedione conjugated with horseradish peroxidase (HRP)
5. **SORB | MT Microtiterwells** (1 microplate breakable)
   Anti-androstenedione antibody adsorbed on microplate
6. **SUB | TMB Substrate Solution** (1 vial, 15 mL)
   H_2O_2-TMB 0.26 g/L (avoid any skin contact)
7. **STOP | SOLN Stop Solution** (1 vial, 15 mL)
   Sulphuric acid 0.15 mol/L (avoid any skin contact)
8. **WASH | SOLN | 50x** 50X Conc. **Wash Solution** (1 vial, 20 mL)
   NaCl 45 g/L; Tween20 55 g/L

3.2 Reagents necessary not supplied

Distilled water

3.3 Auxiliary materials and instrumentation

Automatic dispenser
Microplate reader (450 nm, 620-630 nm)
Saliva Collection Device

Note
Store all reagents at 2 °C - 8 °C in the dark.
Open the bag of reagent 5 (Coated Microplate) only when it is at room temperature and close 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 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 °C - 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 °C - 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 lipaemic 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 Standard

(S0, S1, S2, S3, S4)

Before use, mix for 5 minutes with rotating mixer

The standards are ready to use and have the following concentration of Androstenedione:

	S0	**S1**	**S2**	**S3**	**S4**
pg/mL	0	20	100	400	1000

For samples with Androstenedione concentration greater than 1000 pg/mL dilute the sample (1:2) with S0. Once opened, the standards are stable 6 months at 2 °C - 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 for 3 hours at 22 °C - 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 °C - 8 °C.

6.4 Preparation of the Sample and Controls

This kit allows the determination of Androstenedione concentration in saliva samples.
The controls are ready to use.

6.4.1 Specimen

Samples containing sodium azide should not be used in the assay. The saliva samples should be completely colorless. Even the slightest red color shows blood contamination. Such blood contamination will give falsely elevated concentration values. In case of visible blood contamination the patient should discard the sample, rinse the sampling device with tap water, also rinse the mouth with (preferably) cold water, wait for 10 minutes and take a new sample. Do not chew anything during the sampling period. Any pressure on the teeth may result in falsely elevated measurements due to an elevated content of gingival liquid in the saliva sample.

6.4.2 Specimen Collection

For the correct collection of saliva we are recommending to only use appropriate devices made from ultra-pure polypropylene. Do not use any PE devices or Salivettes for sampling; this in most cases will result in significant interferences. Glass tubes can be used as well, but in this case special attention is necessary for excluding any interference caused by the stopper. Please contact Demeditec Diagnostics for more details.

As food might contain significant amounts of steroid hormones samples preferably should be taken while fasting. If fasting should be a problem at least any food of animal origin (meat or dairy products) should be avoided prior to finalizing the collection. In the morning breakfast should be done only after finalizing the collection procedure. During the day the collection period should be timed just before an anticipated meal. As the steroid hormone secretion in saliva as well in serum shows an obvious dynamic secretion pattern throughout the day it is important to always collect 5 samples during a 2 hour period; this means every 30 minutes one sample. If possible the volume of each single sample should be a minimum of 0.5 ml (better 1 ml). Saliva flow may be stimulated by drinking water. This is allowed and even recommended before and during the collection period. Drinking of water is not allowed during the last 5 minutes before taking the single samples. The typical timing for a morning collection period would be as follows. Wake-up at 6:00 AM, drinking water and brushing teeth, 1st sample at 6:15 AM, followed by samples at 6:45 AM, 7:15 AM, 7:45 AM, and 8:15 AM, followed by breakfast at 8:25 AM. The typical timing for an afternoon collection period would be like: 1st sample at 5:00 PM, followed by samples at 5:30 PM, 6:00 PM, 6:30 PM, 7:00 PM, followed by dinner at 7:10 PM. Modest variation in the collection timing will not be critical, and the collection time-frame can be extended up to 3 hours.

6.4.3 Specimen Storage and Preparation

Saliva samples in general are stable at ambient temperature for several days. Therefore mailing of such samples by ordinary mail without cooling will not create a problem. Storage at 4°C can be done for a period of up to one week. Whenever possible samples preferable should be kept at a temperature of -20°C. Even repeated thawing and freezing is no problem. Each sample has to be frozen, thawed, and centrifuged at least once anyhow in order to separate the mucins by centrifugation. Upon arrival of the samples in the lab the samples have to stay in the deep freeze at least overnight. Next morning the frozen samples are warmed up to room temperature and mixed carefully. Then the samples have to be centrifuged for 5 to 10 minutes. Now the clear colorless supernatant is easy to pipette. If the sample should show even a slight reddish tinge it should be discarded. Otherwise the concentration value most probably will be falsely elevated. Due to the episodic variations of the steroid secretion we highly recommend the strategy of multiple sampling. If such a set of multiple samples has to be tested the lab (after at least one freezing, thawing, and centrifugation cycle) has to mix the aliquots of the 5 single samples in a separate sampling device and perform the testing from this mixture.

6.5 Procedure

Allow all reagents to reach room temperature (22 °C - 28 °C). At the end of the assay, store immediately the reagents at 2 °C - 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 °C - 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 standard curve (S0-S4), two for each control and two for each sample, and one for Blank.

Reagent	**Standard**	**Sample/Control**	**Blank**
Standard S0-S4	**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.			
Substrate Solution	**100 µL**	**100 µL**	**100 µL**
Incubate at room temperature 22 °C - 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.			

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 standard 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 standard curve and of each sample.

8.2 Standard Curve

Plot the mean value of absorbance of the standards (Em) (S0 - S4) against concentration. Draw the best-fit curve through the plotted points. (e.g.: Four Parameter Logistic).

8.3 Calculation of Results

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

9 REFERENCE VALUE

As the values of salivary Androstenedione have a circadian pattern we suggest collecting 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 manufacturer 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 measurements (16x) of two different saliva control in one assay. The within assay variability is ≤ 8.5%.

10.1.2 Inter Assay Variation

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

10.2 Accuracy

The recovery of 50 – 200 – 500 pg/mL 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 Standard 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	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

The Androstenedione free in Saliva ELISA kit (DESLV4780) 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 ELISA kit (DESLV4780)

x = Salimetrics Salivary Androstenedione ELISA kit

11 WASTE MANAGEMENT

Reagents must be disposed off in accordance with local regulations.

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13 TROUBLESHOOTING

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

Too high between-run (CV%)
- incubation conditions not constant (time, temperature)
- controls and samples not dispensed at the same time (with the same intervals) (check pipetting order)
- person-related variation

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