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SALIVARY DHEA ENZYME IMMUNOASSAY KIT

Catalog No. 1-2212, (Single) 96-Well Kit;
1-2212-5, (5-Pack) 480 Wells

Intended Use

The Salimetrics™ DHEA kit is a competitive immunoassay specifically designed and validated for the *in vitro* diagnostic measurement of dehydroepiandrosterone (DHEA) in saliva. This kit may be used as an aid in evaluating the synthesizing function of the adrenal gland. Saliva DHEA accurately reflects the amount of serum DHEA in circulation. Salimetrics has not validated this kit for use with serum or plasma samples. Please read the complete kit insert before performing this assay. For further information about this kit, its application, or the procedures in this insert, please contact the technical service team at Salimetrics or your local sales representative.

Introduction

DHEA, a major secretory product of the adrenal glands with anti-oxidant activity, is a precursor to the synthesis of both estrogenic and androgenic steroids (testosterone). (1-4) Low levels may occur in hypoadrenalism. High levels may occur in conditions such as 21-hydroxylase and 3ß-hydroxysteroid dehydrogenase deficiencies, virilizing adrenal adenoma and carcinoma, and some cases of female hirsutism. (4,5) DHEA has also been found to be related to cases of depression (6,7), schizophrenia (8), bone resorption (9,10), obesity (11), and rheumatoid arthritis (12).

In the blood only 1 to 15% of DHEA is in its unbound or biologically active form. The remaining DHEA is bound to serum proteins. Unbound DHEA enters the saliva via intracellular mechanisms, and the majority of DHEA in saliva is non-protein bound. Salivary DHEA levels are unaffected by salivary flow rate or salivary enzymes (13).

This kit is designed to measure DHEA levels in saliva. The standard is in a saliva-like matrix. In addition, a built-in pH indicator warns the user of acidic or basic samples.

Test Principle

A microtitre plate is coated with rabbit antibodies to DHEA. DHEA in standards and unknowns competes with DHEA linked to horseradish peroxidase for the antibody binding sites. After incubation, unbound components are washed away. Bound DHEA 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 with 2-molar sulfuric acid. Optical density is read on a standard plate reader at 450 nm. The amount of DHEA peroxidase detected is inversely proportional to the amount of DHEA present (14).

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. DHEA values from samples with a pH $\leq$ 4.0 or $\geq$ 9.0 may be artificially inflated or lowered (15).

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 (16,17) 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-DHEA Coated Plate:** A ready-to-use, 96-well microtitre plate pre-coated with rabbit anti-DHEA antibodies in a resealable foil pouch.
2. **DHEA Standard:** 1 mL of DHEA in a saliva-like matrix with a non-mercury preservative, at a concentration of 1000 pg/mL.
3. **DHEA Controls:** Two controls representing high and low levels of DHEA 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 60°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:** 50 µL of a solution of DHEA 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-DHEA 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 12 µL, 50 µL, 100 µL, 150 µL and 18 mL
- Precision multichannel pipette to deliver 50 µL, 150 µL, and 200 µL
- Vortex
- Plate rotator with 0.08-0.17 inch orbit (if unavailable, tap to mix)
- 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
- 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 (18,19).

The preferred method for collecting whole saliva is by unstimulated passive drool. Collection protocols are available on request. **Do not use Salivettes, the Salimetrics Oral Swab (SOS), Sorbettes, cotton, or polyester materials to collect samples**. False readings will result (20). 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.

EXAMPLE ONLY

Must use IFU that is shipped with product.

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. Here is a suggested layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1000 Std	1000 Std	Ctrl H	Ctrl H								
B	400 Std	400 Std	Ctrl L	Ctrl L								
C	160 Std	160 Std	Unk-1	Unk-1								
D	64 Std	64 Std	Unk-2	Unk-2								
E	25.6 Std	25.6 Std	Unk-3	Unk-3								
F	10.2 Std	10.2 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. 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 150 µL of assay diluent into tubes 2 through 6.
  Serially dilute the standard 2.5X by adding 100 µL of the 1000 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, are respectively, 1000 pg/mL, 400 pg/mL, 160 pg/mL, 64 pg/mL, 25.6 pg/mL, and 10.2 pg/mL. Standard concentrations in nmol/L are 3.47, 1.39, 0.55, 0.22, 0.09, and 0.03, respectively.
- Pipette 18 mL of assay diluent into the disposable tube. (Scale down proportionally if using less than 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 1:1500 by adding 12 µL of the conjugate to the 18 mL of assay diluent prepared in Step 3. (Scale down proportionally if using less than the entire plate.) Immediately mix the diluted conjugate solution and add 150 µL to each well using a multichannel pipette.

Step 6: Cover plate with adhesive cover provided. Mix plate on rotator for 5 minutes at 500 rpm (or tap to mix) and incubate at room temperature for 3 hours.

Step 7: Wash the plate 4 times with 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 last 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 (or tap to mix) 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 (or tap to mix). 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 490 to 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.
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.

Limitations

- Samples with DHEA values greater than 1000 pg/mL should be diluted with assay diluent and rerun for accurate results. To obtain the final DHEA concentration, multiply the concentration of the diluted sample by the dilution factor.
- A pH value should be obtained on samples that appear yellow or purple after assay diluent is added and the plate is mixed. Samples with pH values ≥ 9.0 or ≤ 4.0 should be recollected.
- See "Specimen Collection" recommendations to insure proper collection of saliva specimens and to avoid interfering substances.
- Samples collected with sodium azide are unsuitable for this assay.
- Any quantitative results indicating abnormal DHEA levels should be followed by additional testing and evaluation.

Quality Control

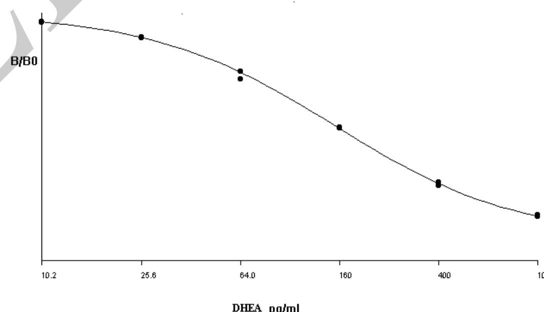
The Salimetrics' high and low salivary DHEA controls should be run with each assay. The control ranges established at Salimetrics are to be used as a guide. Each laboratory should establish its own range. Variations between laboratories may be caused by differences in techniques and instrumentation.

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	DHEA (pg/mL)
A1,A2	S1	0.344	0.295	0.234	1000
B1,B2	S2	0.513	0.464	0.368	400
C1,C2	S3	0.741	0.692	0.548	160
D1,D2	S4	0.958	0.909	0.720	64
E1,E2	S5	1.145	1.096	0.868	23.5
F1,F2	S6	1.23	1.181	0.936	10.2
G1,G2	Bo	1.311	1.262	NA	NA
H1,H2	NSB	0.049	NA	NA	NA

Example: DHEA 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. Correlation with Serum

The correlation between serum and saliva DHEA was determined by assaying 39 matched samples using the Diagnostic Systems Laboratories serum DHEA radioimmunoassay and the Salimetrics Salivary DHEA EIA. The DHEA serum-saliva correlation, using a log 10 transformation for the total (n = 39), combined males and females is 0.857, $p < 0.0001$.

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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 DHEA that can be distinguished from 0 is 5 pg/mL.

C. Precision

1. The intra-assay precision was determined from the mean of 12 replicates each.

Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	Coefficient of Variation (%)
H	12	618.61	32.79	5.3
L	12	44.59	2.58	5.8

2. 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	579.47	45.91	7.9
L	12	34.83	2.97	8.5

D. Linearity of Dilution

Two samples were serially diluted with assay diluent and assayed.

Sample	Dilution Factor	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1			334.66	
	1:2	167.33	157.33	94.0
	1:4	83.67	91.22	109.0
	1:8	41.83	49.44	118.2
	1:16	20.92	20.08	96.0
2			511.38	
	1:2	255.69	287.69	112.5
	1:4	127.84	140.12	109.6
	1:8	63.92	69.30	108.4
	1:16	31.96	34.25	107.2

Linearity is established from 500 to20 pg/mL

E. Antibody Specificity

Compound	Spiked Concentration (ng/mL)	% Cross-reactivity in HS Salivary DHEA EIA
DHEA-S	1000	0.063
Androstenedione	1000	0.0378
17-β Estradiol	1	ND
Estriol	1000	ND
Estrone	1000	ND
Progesterone	1000	ND
17 α-Hydroxyprogesterone	1000	ND
Testosterone	1000	ND
Dihydroxytestosterone	1000	ND
Dianabol	1000	ND
11-Hydroxytestosterone	1000	ND
19-Nortestosterone	1000	ND
Cortisol	1000	ND
Aldosterone	1000	ND
Cortisone	1000	ND
11-Deoxycortisol	1000	ND
21-Deoxycortisol	1000	ND
Triamincinolone	1000	ND
Corticosterone	1000	ND
Transferrin	1000	ND

ND = None detected (<0.004)

F. Recovery

Six saliva samples containing different levels of an endogenous DHEA were spiked with known quantities of DHEA and assayed.

Sample	Endogenous (pg/mL)	Added (pg/mL)	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1	84.45	50	134.45	136.21	98.7
2	66.84	400	466.84	511.34	109.5
3	317.92	50	367.92	334.18	90.8
4	317.92	500	817.92	919.4	112.4
5	41.84	16	57.84	64.49	111.5
6	185.04	16	201.04	181.30	90.2

G. Method Comparison

The correlation between the Salimetrics EIA and a published serum RIA modified for use with saliva (17) was evaluated by assaying 40 common samples. The EIA-RIA results were highly correlated, $r\ (38) = 0.881$, $p < 0.001$.

H. Salivary DHEA Expected Ranges: *

Group	Number	Mean (pg/mL)	Standard Deviation (pg/mL)
Females	19	165.6	71.6
Males	20	153.5	68.8

*To be used as a guide only. Each laboratory should establish its own range.

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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."

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