SPECIFIC SALIVARY DHEA EIA KIT

For Research Use Only

Catalog Number: DHA32-K01

Eagle Biosciences

20A NW BLVD., Suite 112
Nashua, NH 03063
Phone: +1 (617) 419-2019
Fax: +1 (617) 419-1110
Info@eaglebio.com

TABLE OF CONTENTS

I. Intended Use and Description

The Eagle Biosciences Salivary Dehydroepiandrosterone (DHEA) EIA kit is designed and validated for the **specific** quantitative measurement of DHEA in human saliva.

II. Assay Background

DHEA is a C19 steroid produced in the adrenal cortex and to a lesser extent gonads (1). DHEA has a relatively low affinity for sex hormone binding globulin (2). It circulates in the blood as precursors to estrogens and androgens (3). DHEA levels are high at birth, low in children, increases through puberty and reaches peak levels just after puberty (4). In both male and female levels begin to fall as age increases (5). DHEA is cleared more rapidly than dehydroepiandrosterone sulfate (DHEA-S) in blood. For this reason, the DHEA levels are 100-1000 times lower than DHEA-S in blood. Only 1-10% of DHEA circulating in plasma is in its free or biologically active form. The rest is bound to serum proteins. In saliva, DHEA enters via intracellular mechanisms and reflects the level of Free DHEA in plasma (6).

The physiological role of DHEA has not been clearly defined. Many papers have been published on DHEA showing effects on human health and physiology. DHEA has been reported to have the following effects: anti-diabetes, anti-dementia, anti-obesity, anti-cancer, anti-stress, anti-viral, anti-aging and anti-cardiac disease. DHEA may serve as a marker for hyperandrenalcorticism. Hirsute women and adolescents with acne may have elevated levels of DHEA (7,8).

III. Assay Principle

The Salivary Dehydroepiandrosterone (DHEA) EIA kit is based on the competition principal and microplate separation. DHEA calibrators of known concentration and unknown amounts of DHEA in saliva samples compete with a fixed amount of DHEA conjugated to horse radish peroxidase (DHEA-HRP) for binding sites with a rabbit DHEA polyclonal antiserum bound to GARGG (goat anti-rabbit gamma globulin) coated wells of a microplate. After incubation, unbound components are washed away, enzyme substrate solution is added and a blue color formed. This reaction is stopped with an acid solution to produce a yellow color. The optical density is then read at 450 nm. The amount of
DHEA-HRP detected is inversely proportional to the amount of DHEA in a sample.

IV. Reagents Supplied and Reagent preparation

Store all other reagents at 2 to 8°C. Use only reagents supplied with this kit. Do not interchange reagents with different lot numbers. Expiration dates and lot numbers are printed on the labels.

1. **GARGG Plate**: One 96 well microplate (12x8 breakable strip wells) coated with goat anti-rabbit gamma globulin placed in a resealable foil bag with desiccant. One (1) 96 well kit is sufficient for 39 duplicate patient measurements.
2. **Concentrated Stock DHEA (synthetic) solution** in BSA buffer at a concentration of 100 ng/ml (100,000 pg/ml): 1 bottle 0.150 ul.
   Salivary DHEA working calibrators preparation: Dilute the 100,000 pg/ml stock solution 1:100 (1 part 100,000 pg/ml + 99 parts assay buffer) with assay buffer to obtain the highest working calibrator (**1000 pg/ml**) then, dilute serially 1:2.5 (starting with the **1000 pg/ml** calibrator) to obtain the following concentrations of **working calibrators**: **400 pg/ml**, **160 pg/ml**, **64 pg/ml**, **25.6 pg/ml** and **10.2 pg/ml. "0" calibrator** is assay buffer.
   Note: A 1:2.5 dilution is defined as 1 part calibrator in question plus 1.5 parts assay buffer.
3. Assay buffer: 1 bottle, 20 ml.
4. **Stock DHEA (synthetic) Control Concentrate (80 ng/ml (80,000 pg/ml))**: 1 bottle, 0.150 ml. Use for the preparation of **working control #2**.

Working Control # 2 Preparation (Example)

Stock DHEA control concentrate 80,000 pg/ml	Assay Buffer	Dilution	Target	Number of EIA wells per 5 ml volume
0.050 ml (50 ul)	**4.950 ml**	**1:100**	**800 pg/ml**	**100**

Working Control #1 Preparation

DHEA working control #2 (800 pg/mL)	Assay Buffer	Dilution	Target	Number of EIA wells per 5 ml volume
0.125 ml	**4.875 ml**	**1:40**	**20 pg/ml**	**100**

Immediately after use, store the unused portions of the **working calibrators** and the **High** and **Low Controls** at 2-8°C. Discard if not used within 28 days of mixing.

5. **Salivary DHEA EIA rabbit polyclonal Antibody**: 1 bottle, 6 ml. The solution is blue.
6. **Salivary DHEA-Horseradish Peroxidase (HRP) concentrate.:** 1 amber bottle, 0.600 ml. The solution is yellow and light sensitive.
7. **DHEA -Horseradish Peroxidase (HRP) conjugate buffer, pH 7.4**: 1 bottle, 3 ml. Use only for the preparation of the **DHEA-HRP working reagent.**
   DHEA-HRP working reagent preparation: For example, determine the amount of **working DHEA-HRP** needed and dilute 1:5 with conjugate buffer pH 7.4 (#7). Mix 0.5 ml of **DHEA-HRP concentrate** (#6) plus 2.0 ml of **conjugate buffer,** (#7). This is sufficient for 100 EIA wells.
   Note: The **DHEA-HRP working reagent** is light sensitive. Immediately after use, wrap the vial with the unused portion of the **DHEA-HRP working reagent** with aluminum foil or alternatatgely prepare the **DHEA-HRP working reagent** in an amber vial. Store at 2-8°C. Discard if not used within 28 days of mixing.
8. **Wash solution (10X concentrated) EIA #1**: 1 bottle, 50 ml of phosphate buffered saline, pH 7.4. Prior to use dilute 1:10 with deionized water.

9. **Color Development Reagent EIA #1**: 1 amber plastic bottle, 15 ml of Tetramethylbenzidine (TMB) plus hydrogen peroxide. Light sensitive.
10. **Stopping Solution EIA #1:** 1 bottle of a 15 ml mixture of diluted sulfuric and hydrochloric acid solution.

V. Storage and Stability

1. When stored at 2° - 8°C, unopened reagents will retain activity until the expiration date. Do not use reagents beyond this date.
2. Use only reagents supplied with this kit. Do not interchange reagents with different lot numbers.
3. Opened reagents must be stored at 2° - 8°C.
4. Microtiter wells must be stored at 2° - 8°C. Once the foil bag has been opened, care should be taken to reseal tightly.
5. Opened kits retain activity for 28 if stored as described above.
6. Expiration dates and lot numbers are printed on the labels.

VI. Materials Needed But Not Provided

1. Device to dispense very accurately 50 ul of saliva.
2. Multichannel pipettors.
3. Microplate or orbital shaker
4. Vortex Mixer

5. Microplate washer (not required, plates can be washed manually).
6. Microplate reader capable of reading 450 nm with 4 parameter data reduction or comparable software.
7. Plate Sealers
8. Sample collection device

VII. Sample Collection Processing

1. This samples collection and processing procedure must be followed:
   a. A **suitable collection device**, is required for the collection of saliva samples when determining DHEA concentrations with the Specific DHEA Salivary EIA Kit

   b. Avoid food consumption, drinking coffee or alcohol, smoking or chewing gum 15 minutes prior to sample collection.
   c. Rinse mouth thoroughly with water 15 minutes prior to collection.
   d. In the **required saliva collection device** collect a minimum of 1 mL, (Use the number 1 marked on the collection tube as a reference), of whole saliva by un-stimulated passive drool by allowing saliva to drip off the lower lip into the graduated collection tube or by allowing saliva to accumulate in the floor of the mouth and spitting it into the collection tube. Label the sample tube with the following information:
      i. Date and time of sample collection
      ii. Patient's name
      iii. Patient's gender
      iv. Patient's date of birth
   e. The sample(s) should be sent as soon as possible after collection to the testing site, they should remain stable under average shipping conditions, including over weekends and holidays and during hot temperatures. If the sample(s) will not be sent the day of collection, store at 2-8°C until ready to be shipped.
   f. Upon sample's arrival to the testing site, the sample(s) should be kept in the collection device to maintain its integrity and freeze (≤ -15°C or below) until day of assay. On day of assay, thaw samples to facilitate precipitation of mucins. Centrifuge at 1500g for ten minutes. Bring samples to room temperature and assay.

2. Salivary Sample stability

Storage	20-28°C	37°C	2-8°C	≤-15°C (7 freeze/thaw cycles)	≤-15°C (Long term)
Stability	Up to 7 days	Up to 7 days	Up to 7 days	Up to 7 days	Up to 12 months

VIII. Procedure Summary Flow Sheet

Calibrator DHEA Sample I.D. pg/ml (pg/ml)	Calibrator, Control, Sample (ul)	HRP DHEA Working (ul) Reagent	Anti-DHEA (ul)		Diluted 10X wash solution. (ul)		Color Developer (ul)		Stopping solution (ul)	
0	50	25	50	Mix. Incubate for 2 hrs. at Room Temperature, shaking.	300	Wash 3X	125	Mix. Incubate 30 min. at room temperature	125	Mix. Read at 450 nm
10.2	50	25	50		300		125		125	
25.6	50	25	50		300		125		125	
64	50	25	50		300		125		125	
160	50	25	50		300		125		125	
400	50	25	50		300		125		125	
1000	50	25	50		300		125		125	
Control #1	50	25	50		300		125		125	
Control #2	50	25	50		300		125		125	
Sample	50	25	50		300		125		125	

IX. Assay Procedure

1. The calibrators, controls and samples should be tested in duplicate and the mean value should be used to report the results.
2. To the **GARGG microplate** dispense **50ul** of **working Salivary DHEA EIA calibrators (0, 10.2, 25.6, 64, 160, 400 and 1000** pg/ml), **controls**, and **saliva samples.**
3. Add **25 ul** of **DHEA-HRP working reagent** to all wells.
4. Add **50 ul** of **DHEA EIA rabbit polyclonal antibody**.
5. Cover microplate with plastic sealer. Incubate by shaking on a microplate orbital shaker set a 500-900 rpm for **2 hrs.** at room temperature.
6. After incubation, decant the contents of the wells. Wash 3 times with 300 ul of **diluted wash solution.** After the 3rd wash, invert GARGG microplate on an absorbent paper and tap dry.
7. Dispense **125 ul of Color Development reagent EIA #1** into each well. Shake briefly (manual). Cover microplate with plastic sealer. Incubate for **30 minutes** at room temperature.
8. Dispense **125 ul of Stopping Solution EIA #1** into each microtiter well of the **GARGG** plate. Shake briefly (manual). Color changes from blue to yellow.
9. Read at 450 nm on a microplate reader within 10 minutes.

Note: If samples exceed the upper end of the measuring range of 1000 pg/mL, dilute with zero calibrator and make appropriate concentration correction.

X. Typical Results

Typical Calibration Curve. Testing Date 8/21/13			
Calibrators (pg/ml)	Mean Absorbance (450 nM)	% B/Bo	Value (pg/ml)
0	2.61	100.0	0.0
10.2	2.30	88	10.2
25.6	1.90	73	25.6
64	1.49	57	64
160	1.06	41	160
400	0.65	25	500
1000	0.38	15	1000
Control # 1	1.99	76	22.7
Control # 2	0.43	16	818.7
Sample # 1	0.84	32	243.8
Sample # 2	1.18	45	119.7
Sample #3	1.95	75	25.1

XI. Determination of DHEAConcentration

1. Determine the concentrations of the controls and unknowns by interpolation using software Capable of logistics using a 4-parameter sigmoid minus curve fit.

Analytical measuring range (AMR)	10.2 pg/mL– 1000 pg/mL

Conversion: DHEA (pg/mL) x 3.47 = pmol/L

Samples with DHEA values greater than 1000 pg/mL should be diluted 1:10 with zero (0) calibrator and rerun for accuracy. Obtain the final DHEA concentration by multiplying the diluted sample by 10.

XII. Quality Control

The expected values for the controls are stated on the label of each control which are included in the kit. The results can only be accepted if the expected values are met. Follow federal, state and local guidelines for testing quality control materials.

XIII. Expected DHEA Normal Ranges

Saliva samples (AM) collected at approximately the same time show the following values:

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Subjects (Number)	Gender	Age (Years)	Median (pg/mL)	Range (pg/mL)
58	Female	20 – 49	91.0	14.9 – 318.1
40	Female	50 - 70	65.0	13.5 – 390.7
58	Male	20 – 49	257.8	79.1 – 979.0
40	Male	50 - 70	103.2	43.2 – 620.3

It is recommended that each laboratory establishes its own range of normal values.

XIV. Performance Characteristics

A. Specificity of the Antiserum

Steroids	% Cross-reactivity
Dehydroepiandrosterone	100
Dehydroepiandrosterone SO4	0.0037
Testosterone	0.002
5α-Dihydrotestosterone	0.007
Androstenedione	0.056
Progesterone	0.230
17-αHydroxyprogesterone	0.0004
Pregnenolone	0.013
17-OH-Pregnenolone	0.072
Desoxycorticosterone	0.052
Corticosterone	0.004
Cortisol	0.0007
11-Desoxycortisol	0.012
Aldosterone	0.0003
Estradiol-17β	< 0.001
Estradiol-17α	< 0.001
Estrone	< 0.001
Estriol	< 0.001

B. Detection limits

The Detection Limit Study for determining the limit of the blank (LoB), limit of detection (LoD) and the limit of quantitation (LoQ) for the Specific Salivary DHEA EIA Kit was performed using several low DHEA samples and two different reagent lot numbers that were assayed twice per day over a period of 3 days. (Reference, CLSI EP 17-A, protocols for Determination of Limits of Detection and Limits of Quantitation).

Limit of the Blank (LoB) (pg/ml)	Limit of Detection (LoD) (pg/ml)
0.650	1.163

C. Precision and Reproducibility:

Intra-assay

The intra-assay precision was determined from the mean of 20 replicates of low, medium and high pools

Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	%CV
Low	20	27.0	3.448	12.8
Medium	20	333.3	23.000	6.9
High	20	603.9	65.954	10.9

Inter-assay

The inter-assay precision was determined from the mean average of the duplicates for 12 separate assays with low, medium and high pools.

Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	%CV
Low	12	24.7	3.0	12.2
Medium	12	376.4	37.3	9.9
High	12	643.6	56.8	8.8

Inter-lot Variation

The inter-lot precision was determined by duplicate measurements of three (3) saliva pools and three (3) spiked controls in saliva like matrix, using three (3) different reagent lots.

Saliva Samples ID	Lot # 001 mean (pg/ml)	Lot # 002 mean (pg/ml)	Lot # 003 mean (pg/ml)	Inter-lot mean (pg/ml)	Inter-lot Std. Dev. (pg/ml)	Inter-lot CV (%)
Pool 1	51.7	51.8	53.8	52.4	1.185	2.3
Pool 2	424.3	443.1	402.9	423.4	20.114	4.8
Pool 3	230.5	224.0	249.3	234.6	13.139	5.6
Control 1	28.1	27.7	27.0	27.6	0.557	2.0
Control 2	115.8	123.1	126.2	121.7	5.339	4.4
Control 3	525.3	647.3	550.2	574.4	64.632	11.3

D. **Linearity Study:**

Ten (10) sample concentrations that span the assay measuring range were prepared and assayed per EP6-A, Evaluation of the Linearity of Quantitative Measurement Procedures.

S=10 samples (dilutions)

Concentration = (C1*V1 + C10*V10)/(V1+V10)

	C1 pg/mL	V1 mL	C10 pg/mL	V10 mL	Calculated Concentration pg/mL	Observed Concentration pg/mL	Recovery %
1					4.0	3.5	87.5
2	4.0	0.889	1250.0	0.111	142.3	151.1	106.2
3	4.0	0.776	1250.0	0.222	280.6	278.8	99.4
4	4.0	0.667	1250.0	0.333	418.9	428.4	102.3
5	4.0	0.556	1250.0	0.444	557.2	534.8	96.0
6	4.0	0.444	1250.0	0.556	696.8	686.3	98.5
7	4.0	0.333	1250.0	0.667	835.1	824.0	98.7
8	4.0	0.222	1250.0	0.778	973.4	1024.9	105.3
9	4.0	0.111	1250.0	0.889	1111.7	1193.5	107.4
10					1250.0	1238.7	99.1

***** Targets of low and high sample concentrations.

E. Recovery

Five (5) saliva samples containing different levels of 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	844.3	100.0	944.3	915.3	96.9
2	259.7	200.0	459.7	499.8	108.7
3	213.6	400.0	613.6	642.0	104.6
4	129.5	600.0	729.5	752.7	103.2
5	26.0	800.0	826.0	862.5	104.4

XV. Limitations

1. The reagents are optimized to measure DHEA directly in human saliva.
2. Samples containing sodium azide are not suitable for this assay.
3. Avoid blood contamination of samples. Do not collect samples when oral diseases, inflammation or lesions exist.
4. Improper handling of samples or modification of this assay might influence the results.

XVI. Precautions

Only physician, clinical labs, research labs and hospital labs may acquire, possess and use the kit.

1. Compare contents and packing list, if there is breakage or shortage, notify Pantex immediately.
2. Do not pipet reagents by mouth.
3. Do not smoke, eat or drink while performing assay.
4. Wear disposable rubber gloves.
5. Treat all saliva samples as potentially infectious.
6. This kit is for research only. Follow the working instructions carefully.
7. Avoid contact with Color Development Reagent (TMB). It contains solvents that can irritate skin and mucus membranes. If contact is made, wash thoroughly with water.
8. Avoid contact with stopping solution. It contains acid. If contact is made, rinse thoroughly with water.

XVII. References

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