SALIVARY UNCONJUGATED ESTRIOL (FREE ESTRIOL) EIA KIT

For Research Use Only

Catalog Number: DHA32-K01

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I. Intended Use and Description

The Eagle Bio Salivary Free Estriol is designed and validated for the quantitative measurement of free estriol in human saliva.

II. Assay Background

Estriol is the weakest of the three major estrogens with a binding affinity for the estrogen receptor about 1/100 that of estradiol. Physiologically significant amounts of Estriol are only present during pregnancy or with supplementation. Estriol is available, mainly, as a custom compounded product and is one of the components of bi- or tri-estrogen creams usually in a ratio of estradiol to estrone to estriol 1:1:8. Salivary estriol median levels are <2 pg/ml in non-pregnant pre- and post-menopausal women as well as in men. These levels represent little to no physiologic estrogenic activity. In pregnancy, low levels of Estriol may indicate fetal distress and in Down Syndrome Estriol measurements are part of a screen for this condition.

III. Assay Principle

The Salivary Free Estriol ELISA Assay kit is based on the competition principal and microplate separation. Estriol calibrators and unknown amounts of estriol in saliva samples compete with a fixed amount of estriol conjugated to horse radish peroxidase (**Estriol-HRP**) for binding sites with a rabbit estriol monoclonal 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 **Estriol-HRP** detected is inversely proportional to the amount of estriol 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 Estriol (synthetic) solution** in BSA buffer at a concentration of 121.5 ng/ml (121,500 pg/ml): 1 bottle, 150 ul.
   Determine the amount of working estriol calibrators needed and prepare based on this example:

Working Estriol working calibrator 1215 pg/mL preparation:

Calibrator Concentration to prepare (pg/mL)	Stock Estriol Concentrate to use (pg/mL)	Volume to Use (mL)	Assay Buffer to use (mL)	Final Volume (mL)
1215	121,500	0.030	2.970	3.000

Working Estriol Calibrators 405 - 5 pg/mL preparation:

Calibrator Concentration to prepare (pg/mL)	Calibrator Concentration to use (pg/mL)	Volume to use (mL)	Assay buffer to use (mL)	Final Volume (mL)
405	1215	1.000	2.000	3.000
135	405	1.000	2.000	3.000
45	135	1.000	2.000	3.000
15	45	1.000	2.000	3.000
5	15	1.000	2.000	3.000
0			3.000	3.000

3. **Assay buffer**: 1 bottle, 20 ml.
4. **Stock Estriol (synthetic) Control Concentrate (50 ng/ml (50,000 pg/ml))**: 1 bottle, 0.150 ml. Concentration is on the label and is traceable to U.S. Pharmacopeia (USP).
   Determine the amount of working controls needed and prepare based on this example:

Working Estriol Control #2, (500 pg/mL) preparation

Control Concentration to prepare (pg/mL)	Stock Concentration to use (pg/mL)	Volume to use (mL)	Assay Buffer to use (mL)	Final Volume (mL)
500	50,000	0.020	1.980	2.000

Working Estriol Control #1 (25 pg/mL) preparation:

Control Concentration to prepare (pg/mL)	Progesterone Control #2 Concentration to use (pg/mL)	Volume to use (mL)	Assay Buffer to use (mL)	Final Volume (mL)
25	500	0.050	0.950	1.000

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 Estriol EIA rabbit monoclonal Antibody**: 1 bottle, 6 ml. The solution is blue.
6. **Salivary Estriol-Horseradish Peroxidase (HRP) concentrate.:** 1 amber bottle, 0.100 ml. Estriol derivative is conjugated to horseradish peroxidase. The solution is yellow and light sensitive.
7. **Estriol -Horseradish Peroxidase (HRP) conjugate buffer, pH 7.4**: 1 bottle, 3 ml. Use only for the preparation of the **Estriol-HRP working reagent only**.
   Estriol-HRP working reagent preparation: Determine the amount of **working Estriol-HRP** needed and dilute 1:40 with **conjugate buffer** pH 7.4 (#7). For example, mix 0.0625 ml of **Estriol-HRP concentrate** (#6) plus 2.437 ml with **conjugate buffer,** (#7). This is sufficient for 100 EIA wells.
   The **Estriol-HRP working reagent** is light sensitive. Immediately after use, wrap the vial with the unused portion of the **Estriol-HRP working reagent** with aluminum foil or alternatively, prepare the **Estriol-HRP working reagent** in an amber vial. Store at 2-8°C. Discard if not used within 14 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.

*Concentration of estriol calibrators and controls are actual and traceable to US Pharmacopeia (USP) Cat. No. 125408 Lot L0K175

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 days 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. Suitable 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 Estriol concentrations with this 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. 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. Assay Procedure Summary Flow Sheet

Calibrator Estriol Sample I.D. pg/ml	Calibrator, Control, Sample (ul)	HRP Estriol Working (ul) solution	Anti-Estriol (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
5	50	25	50		300		125		125	
15	50	25	50		300		125		125	
45	50	25	50		300		125		125	
135	50	25	50		300		125		125	
405	50	25	50		300		125		125	
1215	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. It is recommended that 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 Estriol EIA calibrators** (0, 5, 15, 45, 135, 405 and 1215 pg/ml), **controls**, and **saliva samples**.
3. Add **25 ul** of **Estriol-HRP working reagent** to all wells.
4. Add **50 ul** of **Estriol EIA rabbit monoclonal 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 500 pg/mL, dilute with zero calibrator and make appropriate concentration correction.

X. Typical Results

Typical Calibration Curve (Actual assay)			
Calibrators (pg/ml)	Mean Absorbance (450 nm)	% B/Bo	Value (pg/ml)
0	2.62	100	0
5	2.12	81.0	5
15	1.66	64.4	15
45	1.01	38.7	45
135	0.43	16.5	135
405	0.18	6.7	405
1215	0.09	3.3	1215
Control # 1	1.41	53.9	22
Control # 2	0.17	6.5	448
Sample # 1	0.12	4.6	650
Sample # 2	0.61	23.3	95
Sample #3	1.99	76.0	11

XI. Determination of Estriol Concentration

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)	5.0 pg/mL– 1215 pg/mL

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

Samples with Estriol values greater than 1215 pg/mL should be diluted 1:10 with zero (0) calibrator and rerun for accuracy. Obtain the final Estriol 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 Estriol Normal Ranges

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

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Subjects (Numbers)	Gender	Median (pg/mL)	Range (pg/mL)
59	Female pre-menopausal	0.8	0.0 – 12.4
40	Female post-menopausal	1.2	0.0 – 18.4
98	Male 20-70 years old	1.6	0.0 – 17.1

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

XIV. Performance Characteristics

A. Specificity of the Antiserum

Steroids	**% Cross-reactivity**
Estriol (E3)	100.000
15-Hydroxestriol	4.4220
16-Epiestriol	1.7400
Estriol-3-SO4	0.0248
Estriol-3-glucoronide	0.0290
Estriol-16-glucoronide	0.0300
Estradiol 17 β (E2)	0.1300
Estradiol 17α	0.2214
Estradiol-3-SO4	<0.001
Estrone (E1)	<0.001
Estrone-3-SO4	0.100
Androstenedione	< 0.001
Testosterone	< 0.001
5α-Dihydrotestosterone	< 0.001
Dehydroepiandrosterone SO4	< 0.001
Androstanedione	< 0.001
Progesterone	< 0.001
17OH Progesterone	< 0.001
Pregnenolone	< 0.001
17-OH-Pregnenolone	< 0.001
Desoxycorticosterone	< 0.001
11-Desoxycorticosterone	< 0.001
Corticosterone	< 0.001
Aldosterone	< 0.001
Cortisol	<0.001

B. Detection limits

The Detection Limit Study for determining the limit of the blank (LoB) and limit of detection (LoD) for the Salivary Unconjugated Estriol EIA Kit was performed using several low estriol 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)
0.500	0.860

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	36.2	2.950	8.2
Medium	20	130.0	6.364	4.9
High	20	388.9	23.68	6.0

Inter-assay

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

Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	%CV
Low	12	36.4	1.966	5.4
Medium	12	141.6	10.029	7.1
High	12	414.6	18.089	4.4

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	82.8	89.9	99.3	90.7	8.277	9.1
Pool 2	185.4	204.1	211.6	200.4	13.493	6.7
Pool 3	6.8	6.3	6.3	6.5	0.289	4.5
Control 1	36.9	37.8	37.6	37.4	0.473	1.3
Control 2	138.6	156.6	141.1	145.4	9.751	6.7
Control 3	426.1	457.3	461.5	448.3	19.340	4.3

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					4.5	4.4	97.8
2	4.5	0.889	1250.0	0.111	142.8	150.4	105.4
3	4.5	0.778	1250.0	0.222	281.0	303.0	107.8
4	4.5	0.667	1250.0	0.333	419.3	429.6	102.5
5	4.5	0.556	1250.0	0.444	557.5	526.1	94.4
6	4.5	0.444	1250.0	0.556	697.0	674.8	96.8
7	4.5	0.333	1250.0	0.667	835.2	803.8	96.2
8	4.5	0.222	1250.0	0.778	973.5	912.8	93.7
9	4.5	0.111	1250.0	0.889	1111.7	1009.1	90.8
10					1250.0	1105.0	88.4

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

E. Recovery

Five (5) saliva samples containing different levels of endogenous estriol were spiked with known quantities of Estriol and assayed.

Sample	Endogenous (pg/ml	Added (pg/ml)	Expected (pg/ml)	Observed (pg/ml)	Recovery (%)
1	6.5	10.0	16.5	14.5	87.9
2	6.8	50.0	56.8	55.7	98.1
3	14.5	500.0	514.5	449.9	87.4
4	4.8	1000.0	1004.6	1099.3	109.4
5	4.8	1250.0	1254.8	1337.1	106.6

XV. Limitations of the Procedure

1. The Salivary Unconjugated Estriol EIA Kit reagents are optimized to measure estriol in human saliva.
2. Avoid the use of samples with blood contamination, sodium azide and thimerosal as it may lead to false results.
3. Salivary Estriol concentrations in pregnant women have not been established with the Salivary Free Estriol EIA Kit. For information covering this topic, refer to references 3 and 4 of the kit insert.

XVI. Precautions

1. Only physician, clinical labs, research labs and hospital labs may acquire, possess and use the kit.
2. This kit is for research only. Follow the working instructions carefully.
3. Do not pipet reagents by mouth.
4. Do not smoke, eat or drink while performing assay.
5. Wear disposable rubber gloves.
6. Treat all saliva samples as potentially infectious.
7. Do not mix reagent lot numbers or alter in any way the reagents in this kit.
8. 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.
9. Avoid contact with stopping solution. It contains acid. If contact is made, rinse thoroughly with water.

XVII. Selected and Cited Product References

References

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