AnshLabs

FOR PROFESSIONAL USE ONLY

Unconjugated Estriol EIA

IVD

AL-138

INTENDED USE

The Unconjugated Estriol Enzyme Immunoassay (EIA) Kit provides materials for the quantitative measurement of unconjugated estriol in serum. This assay is intended for *in vitro Diagnostic Use Only.* The test *may help* aid in the diagnosis and treatment of fetoplacental distress.

SUMMARY AND EXPLANATION

Estriol (1,3,5(10)-estratriene-3,16α,17β-triol; E_3) is one of the 3 major naturally occurring estrogens, the others being estradiol and estrone. Estriol is produced almost exclusively during pregnancy, and is the major estrogen produced in the normal human fetus. During pregnancy, the production of estriol depends on an intact maternal-placental-fetal unit.[1,2] Steroid precursors from the maternal circulation are taken up by the placenta and converted to progesterone. Progesterone is then converted to dehydroepiandrosterone sulfate (DHEA-S) in the fetal adrenal gland, which is then 16α-hydroxylated in the fetal liver. 16α-hydroxylase activity is present in only very low amounts in placenta and non-fetal tissues. In the placenta, 16α-hydroxy-DHEA-S is then converted sequentially to 16α-hydroxy-DHEA, 16α-hydroxyandrost-4-ene-3,17-dione and, finally, estriol. Estriol may also originate from estrogen precursors, such as 16α-hydroxyestrone. This pathway may account for the high levels of estrone sulfate found in breast cyst fluid.[3]

Fetal-placental production of estriol leads to a progressive rise in maternal circulating estriol levels, reaching a late-gestational peak which is 2-3 orders of magnitude greater than non-pregnant levels.[1,2] In the maternal circulation, estriol undergoes rapid conjugation in the liver followed by urinary excretion with a half-life of ~20 minutes.[1] Therefore, maternal estriol levels can provide a dynamic estimate of fetal production rates. In terms of estrogenic activity, estriol is much less potent than estradiol.[2] The physiologic role of estriol is not known.

Specific diagnostic and therapeutic uses for estriol measurements are not completely defined, although clinical utility during pregnancy has been investigated. Since normal estriol production depends on an intact maternal-placental-fetal circulation and functional fetal metabolism, maternal estriol levels have been used to monitor fetal status during pregnancy, particularly during the third trimester. Because estriol concentrations are subject to diurnal and episodic variation, it is common practice to refer serum measurements to a baseline, defined for the patient as either the average or the highest of her three most recent estriol results. However, studies in diabetic pregnancies suggest that low estriol levels have limited value in predicting fetal distress.[4] The AL-138 Unconjugated Estriol EIA uses a rabbit anti-estriol antibody preparation with low cross-reactivity to other natural estrogens and estrogen precursors.

PRINCIPLE OF THE TEST

The Unconjugated Estriol EIA Kit uses the competitive binding enzyme immunoassay format. In the assay, Calibrators, Controls and Unknowns containing unconjugated estriol are incubated with biotin-labeled estriol and rabbit anti-estriol antiserum in microtitration wells coated with goat anti-rabbit gamma globulin where the unlabeled and biotin-labeled antigens compete for a limited number of anti-estriol binding sites. After incubation and washing, the wells are incubated with streptavidin-horse radish peroxidase, which binds to the biotinylated estriol. The unbound streptavidin-HRP is washed, followed by incubation with the substrate tetramethylbenzidine (TMB). An acidic stopping solution is then added and the degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 and 620 nm.

MATERIALS SUPPLIED

CAL-138A - CAL-138F Estriol Calibrators

Six vials, 2 mL of Calibrator A and 0.5 ml of Calibrators B-F, containing concentrations of approximately 0 – 11.2 ng/mL Estriol in Estriol calibrator matrix. Refer to **calibration card** for exact concentrations. **Calibrators are shipped ambient. Store at - 20°C upon receipt until the expiration date.** Avoid repeated freeze thaws.

CTR-138-I and CTR-138-II Estriol Controls

Two vials, 0.5 mL each, labeled Levels I and II containing low and high Estriol in Estriol calibrator matrix. Refer to **calibration card** for exact concentrations. **Controls are shipped ambient. Store at - 20°C upon receipt until the expiration date**. Avoid repeated freeze thaws.

AG-002 Goat Anti-Rabbit IgG (GARG) Microtitration Strips

One stripholder, containing 96 polystyrene microtitration wells with Goat anti-rabbit IgG antibody immobilized to the inside wall of each well. Store at 2 to 8°C until expiration date in the resealable pouch with a desiccant to protect from moisture.

BCC-138 Estriol Biotin Conjugate Concentrate

One bottle, 0.4 mL, containing a protein-based buffer with a non-mercury preservative. Store at 2 to 8°C until expiration date.

CND-138 Estriol Conjugate Diluent

One bottle, 12 mL, containing a protein-based buffer with a non-mercury preservative. Store at 2 to 8°C until expiration date.

ABS-138 Anti-Estriol Antibody Solution

One bottle, 12 mL, containing anti-Estriol rabbit polyclonal antibody in a protein-based buffer with a non-mercury preservative. Store at 2 to 8°C until expiration date.

SAR-138 Estriol Streptavidin Conjugate Ready-to-Use

One bottle, 12 mL, containing streptavidin-HRP (horseradish peroxidase) in a protein-based buffer and a non-mercury preservative. Store undiluted at 2-8°C until expiration date.

TMB-100 TMB Chromogen Solution

One bottle, 12 mL, containing a solution of tetramethylbenzidine (TMB) in buffer with hydrogen peroxide. Store at 2 to 8°C until expiration date.

WSH-100 Wash Concentrate A

One bottle, 60 mL, containing buffered saline with a nonionic detergent. Store at 2 to 30°C until expiration date. Dilute 25-fold with deionized water prior to use.

STP-100 Stopping Solution

One bottle, 12 mL, containing 0.2 M sulfuric acid. Store at 2 to 30°C until expiration date.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Microtitration plate reader capable of absorbance measurement at 450 nm, 405nm and 630 nm.
2. Microtitration orbital plate shaker.
3. Microtitration plate washer.
4. Semi-automated/manual precision pipette to deliver 10–250 µL.
5. Repeater pipette
6. Repeater plus for repeated dispensing
7. Vortex mixer.
8. Deionized water.

WARNINGS AND PRECAUTIONS

***For in vitro Diagnostic Use Only*.**

The following precautions should be observed:

a) Follow good laboratory practice.

b) Use personal protective equipment. Wear lab coats and disposable gloves when handling immunoassay materials.

c) Handle and dispose of all reagents and material in compliance with applicable regulations.

WARNING: Potential Biohazardous Material

This reagent may contain some bovine and rabbit antiserum or materials used in conjunction with animal source materials. Handle all reagents and patient samples at a Biosafety Level 2, as recommended for any potentially infectious human material in the Centers for Disease Control/National Institutes of Health manual "Biosafety in Microbiological and Biomedical Laboratories," 5th Edition, 2007[5].

WARNING: Potential Chemical Hazard

Some reagents in this kit contain Pro-Clean 400 and sodium azide[6] as a preservative. Pro-Clean 400 and sodium azide in concentrated amounts are irritants to skin and mucous membranes.

For further information regarding hazardous substances in the kit, please refer to the MSDS, either at AnshLabs.com or by request.

SAMPLE COLLECTION AND PREPARATION

- Serum is the recommended sample type.
- Sample handling, processing, and storage requirements depend on the brand of blood collection tube[6] that you use. Please reference the manufacturer's instructions for guidance. Each laboratory should determine the acceptability of its own blood collection tubes and serum separation products.
- Samples may be stored at 4°C if assayed within 7 days; otherwise samples must be stored at -20°C or -80°C to avoid loss of bioactivity and contamination.
- Avoid assaying lipemic, hemolyzed or icteric samples.
- Avoid repeated freezing and thawing of samples. Thaw samples no more than 3 times.
- For shipping, place specimens in leak proof containers in biohazard specimen bags with appropriate specimen identification and test requisition information in the outside pocket of the biohazard specimen bag. Follow DOT and IATA requirements when shipping specimens.

PROCEDURAL NOTES

1. A thorough understanding of this package insert is necessary for successful use of the Estriol EIA assay. It is the responsibility of the customer to validate the assay for their use. Accurate results will only be obtained by using precise laboratory techniques and following the package insert.
2. A calibration curve must be included with each assay.
3. Bring all kit reagents to room temperature before use. Thoroughly mix the reagents before use by gentle inversion. Do not mix different lots of any kit component and do not use any component beyond the expiration date.
4. Use a clean disposable pipette tip for each reagent, calibrator, control, or sample. Avoid microbial contamination of reagents, contamination of the substrate solutions with the SHRP conjugate. The enzyme used as the label is inactivated by oxygen, and is highly sensitive to microbial contamination, sodium azide, hypochlorous acid and aromatic chlorohydrocarbons often found in laboratory water supplies. Use deionized water.
5. Incomplete washing will adversely affect the outcome and assay precision. Care should be taken to add TMB into the wells accurately and efficiently to minimize potential assay drift due to variation in the TMB incubation time. Avoid exposure of the reagents to excessive heat or direct sunlight.

PREPARATION OF REAGENTS

1. **Wash Solution:** Dilute wash concentrate 25-fold with deionized water. The wash solution is stable for one month at room temperature when stored in a tightly sealed bottle.
2. **Microtitration Wells:** Select the number of coated wells required for the assay. The remaining unused wells should be placed in the resealable pouch with a desiccant. The pouch must be resealed to protect from moisture.
3. **Estriol Biotin Conjugate Solution:** The Estriol Biotin Conjugate Concentrate should be diluted at a ratio of 1 part conjugate to 50 parts of Estriol Conjugate Diluent, according to the number of wells used. If an entire plate is to be used pipet exactly 220 µL of the Concentrate into 11 mL of the diluent.

   Note: Estriol biotin conjugate *concentrate should be freshly diluted 10 minutes prior to use.*

ASSAY PROCEDURE

Allow all specimens and reagents to reach room temperature (23 ± 2°C) and mix thoroughly by gentle inversion before use. Calibrators, controls, and unknowns should be assayed in duplicate.

NOTE: All serum samples reading higher than the highest calibrator should be mixed and diluted in the 0 ng/mL Calibrator A prior to assay.

1. Prepare the **Estriol-Biotin Conjugate Solution** by diluting the Estriol Biotin Conjugate Concentrate in Estriol Conjugate Diluent as described under the Preparation of the Reagents section
2. Label the microtitration strips to be used.
3. Pipette **50 µL** of the **Calibrators** and **Controls** to the appropriate wells.
4. Pipette **25 µL** of the **Calibrator A** to the remaining wells which will be used**.** Pipette **25 µL** of **Unknowns** in the wells containing Calibrator A.
5. Add **100 µL** of **Estriol-Biotin Conjugate Solution** to each well using a repeater pipette.
6. Add **100 µL** of **Estriol Antibody Solution** to each well using a repeater pipette.

7. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **30 minutes** at room temperature (23 ± 2°C).
8. Aspirate and wash each strip 5 times with Washing Solution (350 µL/per well) using an automatic microplate washer.
9. Add **100 µL** of the **Streptavidin-Enzyme Conjugate-RTU** to each well using a repeater pipette.
10. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **30 minutes** at room temperature (23 ± 2°C).
11. Aspirate and wash each strip 5 times with the Wash Solution (350 µL/per well) using an automatic microplate washer.
12. Add **100 µL** of the **TMB chromogen solution** to each well using a repeater pipette. Avoid exposure to direct sunlight.
13. Incubate the wells, shaking at **600–800 rpm** on an orbital microplate shaker, for **10-12 min** at room temperature (23 ± 2°C).
    NOTE: *Visually monitor the color development to optimize the incubation time.*
14. Add **100 µL** of the **Stopping solution** to each well using a repeater pipette. Read the absorbance of the solution in the wells within **20 minutes**, using a microplate reader set to **450 nm.**
    NOTE: *If instrument has a wavelength correction, set the instrument to dual wavelength measurement at **450 nm** with background wavelength correction at **630 nm**.*

RESULTS

1. Optimum results can be obtained at incubation temperature of **23 ± 2°C**.
2. Calculate the mean OD for each calibrator, control, and sample.
3. Plot the mean OD readings for each of the Calibrators along the y-axis versus log of Estriol calibrator concentrations in ng/mL on the x-axis, using a 4PL or sigmoid curve fit.
4. Determine the Estriol concentrations of the controls and samples from the calibration curve by matching their mean OD readings with the corresponding Estriol concentrations.
5. Any sample reading higher than the highest calibrator should be appropriately diluted into a low reading serum sample and re-assayed.
6. Any sample reading lower than the analytical sensitivity should be reported as such.

LIMITATIONS

The reagents supplied in this kit are optimized to measure Estriol levels in Estriol calibrator matrix. If there is evidence of microbial contamination or excessive turbidity in a reagent, discard the vial. For assays employing antibodies, the possibility exists for interference by heterophile antibodies in the samples.[7]

As with all diagnostic tests, a definite clinical diagnosis **should NOT** be based on the results of **a single test**. The diagnosis should only be made by the physician after reviewing all clinical and laboratory findings.

QUALITY CONTROL

- Each laboratory should establish mean values and acceptable ranges to assure proper performance.
- Estriol EIA controls or other commercial controls should fall within established confidence limits.
- The confidence limits for Estriol EIA controls are printed on the calibration card.
- A full calibration curve, low and high level controls, should be included in each assay.
- The TMB chromogen solution should be colorless. Development of a blue color may indicate reagent contamination or instability.

REPRESENTATIVE CALIBRATION CURVE DATA

Well Number	Well Contents Calibrators	Mean Absorbance	Conc (ng/mL)
A1, A2	A	3.06	0.0
B1, B2	B	2.50	0.053
C1, C2	C	1.80	0.25
D1, D2	D	1.07	0.84
E1, E2	E	0.60	2.24
F1, F2	F	0.28	11.2

CAUTION: *The above data must not be employed in lieu of data obtained by the user in the laboratory.*

ANALYTICAL CHARACTERISTICS

All analytical characteristics are stated ng/mL.

Conversion Factor: ng/mL x 3.5 = nmol/L

Analytical Sensitivity:

The analytical sensitivity in the assay as calculated by the interpolation of mean plus two standard deviation of 16 replicates of calibrator A (0 ng/mL) and calibrator B (0.053 ng/mL) is 0.04 ng/mL.

Imprecision:

Reproducibility of the Estriol EIA assay was determined in a study using three serum pools. The study included a total of 5 assays, three replicates of each per assay (n=15). Representative data were and are presented in the following table.

Sample	Mean Conc.	Within Run		Between Run		Total	
	(ng/mL)	SD	%CV	SD	%CV	SD	%CV
Pool-1	0.75	0.04	5.80	0.03	4.38	0.06	7.26
Pool-2	2.60	0.17	6.34	0.13	5.17	0.21	8.19
Pool-3	7.23	0.77	10.63	0.52	7.16	0.93	12.81

Recovery:

Known amounts of Estriol were added to five serum samples containing different levels of endogenous Estriol. The concentration of Estriol was determined before and after the addition of exogenous Estriol and the percent recovery was calculated.

Sample ID	Endogenous Value in ng/mL	Expected in ng/mL	Observed in ng/mL	%Recovery
S1	1.5180	2.322 3.126 3.930	2.562 3.386 4.240	110% 108% 108%
S2	1.3470	2.160 2.972 3.785	2.321 3.008 3.296	107% 101% 87%
S3	1.1840	2.005 2.826 3.646	2.235 3.084 3.873	111% 109% 106%
S4	0.8960	1.731 2.566 3.402	1.741 2.328 3.032	101% 91% 89%
S5	1.6460	2.444 3.241 4.039	2.626 3.293 4.400	107% 102% 109%

Linearity:

Based on CLSI EP6P-A multiple dilutions of the two serum samples containing various Estriol levels and Calibrator F were diluted with calibrator A Matrix. The % recovery on individual samples is represented in the following table.

Sample ID	Dilution factor (1 in X)	Expected Value in ng/mL	Observed Value in ng/mL	Average %Recovery
CAL F	**NEAT VALUE**	11.200		99%
	2	5.600	4.981	
	4	2.800	2.716	
	8	1.400	1.484	
	16	0.700	0.732	
	32	0.350	0.349	
S1	**NEAT VALUE**	6.137		110%
	2	3.069	3.051	
	4	1.534	1.643	
	8	0.767	0.892	
	16	0.384	0.430	
	32	0.192	0.217	
S2	**NEAT VALUE**	4.357		102%
	2	2.179	2.191	
	4	1.089	1.219	
	8	0.545	0.592	
	16	0.272	0.274	
	32	0.136	0.123	

Analytical Specificity:

This monoclonal antibody pair used in the assay detects Estriol. Other related molecules at 100 ng/mL and their % cross-reactivity are represented in the following table.

Cross-Reactant	Concentration (ng/mL)	% Cross-Reactivity
Estrone	100	ND
Estradiol	100	0.822%
Estrone sulphate	100	ND
17-α-ethynyl estradiol	100	ND
β-Estradiol 17-(β-D-glucuronide) Sodium salt	100	0.045%
Aldosterone	100	ND
Androsterone	100	ND
Androstenediol	100	ND
DHEA	100	ND
Progesterone	100	ND
Testosterone	100	ND

ND= Non Detectable (Less than LOD)

Interference:

When hemoglobin and triglycerides were added at a greater than two folds of their physiological concentration to control sample, Estriol concentrations were within ± 20% of the control as represented in the following table. This study was based on CLSI EP7-A2.

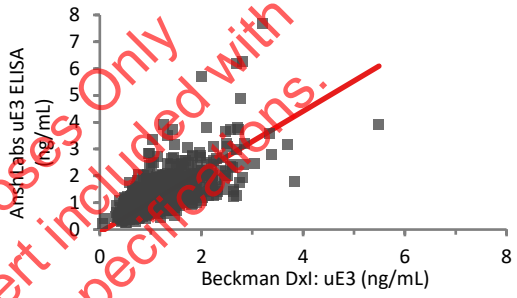
Interferents	Analyte Conc. (mg/mL)	Unspiked Sample Value (ng/mL)	Spiked Sample Value (ng/mL)	% Difference
Hemoglobin	1 mg/mL	6.01	5.42	-9.82
	0.5 mg/mL	6.35	6.53	2.83
	0.1 mg/mL	6.75	6.65	-1.39
Hemoglobin	1 mg/mL	2.87	3.19	11.01
	0.5 mg/mL	2.63	2.60	-0.93
	0.1 mg/mL	2.84	3.01	5.96
Biotin	1200 ng/mL	5.96	5.79	-2.84
	600 ng/mL	7.10	7.42	4.57
	200 ng/mL	5.74	5.96	3.87
Biotin	1200 ng/mL	2.47	2.67	8.39
	600 ng/mL	2.72	2.72	-0.22
	200 ng/mL	3.21	3.29	2.56
Intralipids	20 mg/mL	5.24	4.97	-5.11
	10 mg/mL	5.58	5.81	4.16
	5 mg/mL	5.14	5.25	2.13
Intralipids	20 mg/mL	2.15	2.19	1.84
	10 mg/mL	2.32	2.25	-3.31
	5 mg/mL	2.62	2.46	-6.35
Bilirubin	0.66 mg/mL	3.13	3.31	5.54
	0.2 mg/mL	5.16	5.95	15.38
	0.1 mg/mL	5.86	5.53	-5.80
Bilirubin	0.66 mg/mL	1.76	1.95	11.16
	0.2 mg/mL	2.08	2.24	7.95
	0.1 mg/mL	2.38	2.38	0.20

Method Comparison:

The Estriol EIA has been compared to a commercially available Estriol EIA kit (Method A) using 830 second trimester pregnancy serum samples. Passing Bablok analysis of the results yielded the following Regression:

Estriol EIA (AL-138) = -0.1024 + 1.129 (Beckman Dxl)

(r=0.7; P<0.0001)



Expected Value:

Serum samples were analyzed using Ansh Estriol EIA. The expected ranges for Estriol were calculated using 95% non-parametric estimation using Analyse-It® for Microsoft Excel.

Population	No of specimens (n)	Median conc. (ng/mL)	Estriol 95th (ng/mL)
Gestation Age			
22 Weeks	9	2.79	1.25 – 7.67
21 Weeks	12	2.10	1.37 – 4.89
20 Weeks	26	2.0	1.12 – 3.78
19 Weeks	48	1.91	0.68 – 4.85
18 Weeks	108	1.40	0.65 – 3.18
17 Weeks	158	1.16	0.56 – 2.80
16 Weeks	259	0.94	0.429 – 2.42
15 Weeks	229	0.77	0.36 – 1.54

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This assay is intended for ***in vitro Diagnostic Use Only***.

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