Unconjugated Estriol EIA
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 Research 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; E3) 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 estrenone sulfated 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. 4 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. 5 The AL-138 Unconjugated Estriol EIA uses a rabbit anti-estriol antibody preparation with low cross-reactivity to other natural estrogens and estrogen precursors.

PRINCIPLET 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

<table>
<thead>
<tr>
<th>CAL-138A - CAL-138F</th>
<th>Estriol Calibrators</th>
</tr>
</thead>
<tbody>
<tr>
<td>Six vials, 2 mL of Calibrator A and 0.5 mL of Calibrators B-F, containing concentrations of approximately 0 - 15 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.</td>
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<thead>
<tr>
<th>CTR-138-I and CTR-138-II</th>
<th>Estriol Controls</th>
</tr>
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<tbody>
<tr>
<td>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.</td>
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<thead>
<tr>
<th>AG-002</th>
<th>Goat Anti-Rabbit IgG (GARG) Microtitration Strips</th>
</tr>
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<tbody>
<tr>
<td>One strip holder, containing 96 polystyrene microtitration wells with Goat anti-rabbit IgG antibody immobilized to the inside wall of each well. Store 2 to 8°C until expiration date in the resealable pouch with a desiccant to protect from moisture.</td>
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<table>
<thead>
<tr>
<th>BCC-138</th>
<th>Estriol Biotin Conjugate Concentrate</th>
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<tbody>
<tr>
<td>One bottle, 0.4 mL, containing a protein-based buffer with a non-mercury preservative. Store at 2 to 8°C until expiration date.</td>
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<thead>
<tr>
<th>CND-138</th>
<th>Estriol Conjugate Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>One bottle, 12 mL, containing a protein-based buffer with a non-mercury preservative. Store at 2 to 8°C until expiration date.</td>
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<table>
<thead>
<tr>
<th>ABS-138</th>
<th>Anti-Estriol Antibody Solution</th>
</tr>
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<tbody>
<tr>
<td>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.</td>
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<thead>
<tr>
<th>SAR-138</th>
<th>Estriol Streptavidin Conjugate Ready-to-Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>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.</td>
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<thead>
<tr>
<th>TMB-100</th>
<th>TMB Chromogen Solution</th>
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<tbody>
<tr>
<td>One bottle, 12 mL, containing a solution of tetramethylbenzidine (TMB) in buffer with hydrogen peroxide. Store at 2 to 8°C until expiration date.</td>
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<thead>
<tr>
<th>WSH-100</th>
<th>Wash Concentrate A</th>
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<tbody>
<tr>
<td>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.</td>
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</table>
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, 405 nm 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.
The following precautions should be observed:
a) Follow good laboratory practice.
b) Use personal protective equipment. Wear lab coats and disposable gloves when handling immunocassay materials.
c) Handle and dispose of all reagents and material in compliance with applicable regulations

WARNING: Potential Biohazardous Material
This reagent may contain some human source material (e.g. serum) or materials used in conjunction with human 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, 20076.

WARNING: Potential Chemical Hazard
Some reagents in this kit contain Pro-Clean 400 and sodium azide 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 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. Estriol 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 microtitation 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 3 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 Estradiol calibrator concentrations in ng/mL on the x-axis, using a 4PL or sigmoid curve fit.
4. Determine the Estradiol concentrations of the controls and samples from the calibration curve by matching their mean OD readings with the corresponding Estradiol 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 Estradiol levels in Estradiol 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.

QUALITY CONTROL
- Each laboratory should establish mean values and acceptable ranges to assure proper performance.
- Estradiol EIA controls or other commercial controls should fall within established confidence limits.
- The confidence limits for Estradiol 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.

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

REPRESENTATIVE CALIBRATION CURVE DATA

<table>
<thead>
<tr>
<th>Well Number</th>
<th>Well Contents</th>
<th>Calibrators</th>
<th>Mean Absorbance</th>
<th>Conc (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1, A2</td>
<td></td>
<td>A</td>
<td>2.44</td>
<td>0.0</td>
</tr>
<tr>
<td>B1, B2</td>
<td></td>
<td>B</td>
<td>2.075</td>
<td>0.075</td>
</tr>
<tr>
<td>C1, C2</td>
<td></td>
<td>C</td>
<td>1.313</td>
<td>0.3</td>
</tr>
<tr>
<td>D1, D2</td>
<td></td>
<td>D</td>
<td>0.718</td>
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</tr>
<tr>
<td>E1, E2</td>
<td></td>
<td>E</td>
<td>0.405</td>
<td>3</td>
</tr>
<tr>
<td>F1, F2</td>
<td></td>
<td>F</td>
<td>0.207</td>
<td>15</td>
</tr>
</tbody>
</table>

REFERENCES
This assay is intended for *Research Use Only*.

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Manufactured by:
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U.S.A.