4.0 REAGENTS

A. uE3 Calibrators – 1ml vial - Ions A-F

Six (6) vials of serum or plasma containing unconjugated estriol at concentrations of 0 (A), 0.4 (B), 2.0 (C), 5.0 (D), 15.0 (E), and 30.0 (F) in ng/ml. Store at 2-8°C. A preservative has been added. The calibrators can be expressed in molar concentrations (nmol/L) by the conversion factor 3.45. For example: 1ng/ml = 3.45 x 10^-9 mol/L.

B. uE3 Enzyme Reagent – 6.0 ml - Icon

One (1) vial contains 1mg of rabbit anti-human IgG conjugated to horseradish peroxidase (HRP) conjugated in a protein stabilizing matrix with red dye. Store at 2-8°C.

C. uE3 Biotin Reagent – 6.0 ml - Icon

One (1) vial contains anti-unconjugated Estriol biotinylated purified rabbit IgG conjugate in buffer, blue dye and preservative. Store at 2-8°C.

D. Streptavidin Coated Plate – 96 wells – Icon

One (1) vial contains 20µg/mg of streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

E. TMB Substrate – 5ml vial - Icon

One (1) vial contains tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.

F. STOP Solution – 8ml vial - Icon

One (1) vial contains hydrogen peroxide (H₂O₂) in buffer. Store at 2-8°C.

I. Product Instructions

Note 1: Do not use reagents beyond the kit expiration date.
Note 2: Avoid extended exposure to heat and light. Opened reagents are stable for sixty (60) days when stored at 2-8°C. Kit and component stability are identified on the label.

Note 3: Above reagents are for a single 96-well microplate.

4.1 Required But Not Provided:

1. Pipette capable of delivering 0.025ml (25µl) and 0.050ml (50µl) with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100ml (100µl) and 0.350ml (350µl) volumes with a precision of better than 1.5%.
3. Adjustable volume (200-1000µl) dispenser(s) for conjugate.
4. Microplate washer or a squeeze bottle (optional).
5. Microplate Reader with 450nm and 620nm wavelength absorbance capability.
6. Pipette (flat bottom) for pipetting the microplate wells.
7. Plastic wrap or microplate cover for incubation steps.

8. Vacuum aspirator (optional) for wash steps.
10. Quality control materials.

5.0 PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

All products that contain human serum have been found to be non-reactive for Hepatitis B Surface Antigen, HIV 1&2 and Hepatitis C Antibodies by FDA required tests. Since no known test can offer complete assurance that infectious agents are absent, all human serum products should be handled as potentially hazardous and capable of transmitting disease. Good laboratory procedures for handling blood products can be found in the Center for Disease Control / National Institute of Health, "Biologicals in Microbiological and Biomedical Laboratories," 2nd Edition, 1989, HHS Publication No. (CDC) 88-8395. Safe Disposal of kit components must be according to local regulatory and statutory requirement.

6.0 SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood, serum or heparinised plasma in type and taken with the usual precautions in the collection of venipuncture samples. The blood should be collected in a red (with or without gel additives) venipuncture tube or for plasma use evacuated tube(s) containing heparin. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.

In patients receiving therapy with high biotin doses (i.e. >5mg/day), no sample should be taken until at least 8 hours after the last biotin administration, preferably overnight to ensure fasting sample.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days using one of contaminated devices. Avoid excessive freezing and thawing. When assayed in duplicate, 0.050ml (50µl) of the specimen is required.

7.0 QUALITY CONTROL

Each laboratory should assure controls at levels in the low, normal and high range for monitoring assay performance. These controls should be run with every specimen. It is recommended in every test procedure performed. Quality control charts should be maintained for the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. The individual laboratory should set acceptable assay variability limits. Each lot of control material must be consistent with past experience. Significant deviation from these limits should be reported to the manufacturer. If a specimen is non-reactive or non-conjugate for Hepatitis B Surface Antigen, HIV 1&2 and Hepatitis C antibodies.

Note: Dilute the sample, suspected of concentrations higher than 30ng/ml by diluting 1:2 and/or 1:5 with unconjugated estriol 0’ ng/ml calibrator or male patient sera with a known low value for estriol concentration. Store at 2-8°C for up to 60 days.

8.0 REAGENT PREPARATION

1. Wash Buffer

Dilute contents of wash solution to 1000ml with distilled or deionized water in a suitable storage container. Diluted buffer can be stored at 2-30°C for up to 60 days.

2. Working Substrate Solution - Stable for 1 year

Prepare one vial of substrate Solution A into the vial labeled Solution A'. Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. Store at 2 - 8°C.

Note! Do not use the working substrate if it looks blue.

Note! Do not use reagents that are contaminated or have bacteria growth.

9.0 TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20-27°C). **Test Procedure should be performed by a skilled individual or trained professional**

1. Format the microplates’ wells for each calibrator, control and patient specimen to be assayed. Follow the list of instructions below. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.

2. Pipette 0.025ml (25µl) of the appropriate calibrator, control or specimen into the well assigned.

3. Add 0.050ml (50µl) of the uE3 Enzyme Reagent to all wells except those of a blank.

4. Swirl the microplate gently for 20-30 seconds to mix.

5. Add 0.050ml (50µl) of Wash Buffer to all wells.

6. Swirl the microplate gently for 20-30 seconds to mix.

7. Cover and incubate for 60 minutes at room temperature.

8. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.

9. Add 0.350ml (350µl) of wash buffer (see Reagent Preparation Section), decant and blot again. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used. Follow the manufacturer’s instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.

10. Add 0.100ml (100µl) of working substrate solution to all wells (see Reagent Preparation Section). Always add reagents in the same order to minimize reaction time differences between wells.

DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION

11. Incubate at room temperature for fifteen (15) minutes.

12. Add 0.050ml (50µl) of stop solution to each well and gently mix for 15-20 seconds. Always add reagents in the same order to minimize reaction time differences between wells.

13. Read the absorbance in each well at 450nm (using a reference wavelength of 630-650nm). The results should be read immediately after addition of stop solution.

Note: Dilute the sample, suspected of concentrations higher than 30ng/ml by diluting 1:2 and/or 1:5 with unconjugated estriol 0’ ng/ml calibrator or male patient sera with a known low value for estriol concentration. Store at 2-8°C for up to 60 days.

14. Plot the absorbance for each duplicate serum reference versus the corresponding unconjugated estriol concentration in ng/ml and determine the result.

15. Connect the points with a best-fit curve.

16. To determine the concentration of unconjugated estriol for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in ng/ml) from the horizontal axis of the graph. The duplicates of the unknown may be averaged as indicated. In the following equation, A is the absorbance of the unknown, and X is the concentration (in ng/ml) of unconjugated estriol.

17. Note! Computer data reduction software designed for ELISA analysis is recommended for data reduction. If such software is utilized, the validation of the software should be ascertained.
10. Accurate and precise pipetting, as well as following the exact
11. All applicable national standards, regulations and laws,
8. Use components from the same lot. No intermixing of reagents
7. Failure to remove adhering solution adequately in the

**12. Interpretation**
1. Measurements and interpretation of results must be performed by a skilled individual or trained professional.
2. Laboratory results alone are only one aspect for determining patient care and should rest on the basis of therapy, particularly if the results conflict with other determinants.
3. The reagents for the test system have been formulated to eliminate maximal interference. However, potential interaction between rare serum specimens and test reagents can cause erroneous results. Hormetic antibodies often cause these interactions and it has been accepted to be problematic for all kinds of immunoassays (Bosco LA, STM MC. ‘Hormetic antibodies: a problem for ‘immunoassays’ Clin. Chem. 1988:3427-33). For diagnostic purposes, the results from this assay should be in combination with clinical examination, patient history and all other clinical findings. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
4. If test kits are altered, such as by mixing parts of different kits, which could produce false test results, or if results are incorrectly interpreted, Monobind shall have no liability. If computer controlled data reduction is used to interpret the results of the test, it is imperative that the predicted values for the calibrators fall within 10% of the assigned concentrations.
9. The absorbance (OD) of calibrator 0 ng/ml should be > 1.3

**13.0 EXPECTED RANGES OF VALUES**
In agreement with established reference intervals4 for a “normal” adult population, the expected ranges for the Unconjugated Estriol AccuBind® ELISA Test System are detailed in Table 1.

**TABLE 1**

<table>
<thead>
<tr>
<th>Expected Values for the uE3 ELISA Test System (ng/ml)</th>
<th>Male &amp; Non-Pregnant Female</th>
<th>&lt; 1.0 ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>When pregnant the Unconjugated E3 serum levels rise rapidly till the end of third trimester. (See Table 2 from published literature)5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 2**

<table>
<thead>
<tr>
<th>Estimated Range (ng/ml)</th>
<th>Gestation Week</th>
<th>Estimated Range (ng/ml)</th>
<th>Gestation Week</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 0.3-1.0</td>
<td>22 2.7-16.0</td>
<td>14 0.4-1.6</td>
<td>26 3.0-18.0</td>
</tr>
<tr>
<td>16 1.4-6.5</td>
<td>32 4.6-20.0</td>
<td>18 1.6-8.5</td>
<td>36 7.2-20.0</td>
</tr>
<tr>
<td>20 2.1-13.0</td>
<td>40 8.6-39.0</td>
<td>15.0: 26.1 - 28.9</td>
<td></td>
</tr>
</tbody>
</table>

**14.0 PERFORMANCE CHARACTERISTICS**

**14.1 Precision**

The within and between assay precision of the uE3 AccuBind® ELISA Test System were determined by analyses on three different levels of pool control sera. The number, mean values, standard deviation and coefficient of variation for each of these control sera are presented in Table 3 and Table 4.

**TABLE 3**

<table>
<thead>
<tr>
<th>Sample</th>
<th>X</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>1.58 0.13</td>
<td>8.3%</td>
</tr>
<tr>
<td>Normal</td>
<td>5.17 0.37</td>
<td>7.1%</td>
</tr>
<tr>
<td>High</td>
<td>9.06 0.59</td>
<td>6.5%</td>
</tr>
</tbody>
</table>

**TABLE 4**

<table>
<thead>
<tr>
<th>Sample</th>
<th>X</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>1.04 0.14</td>
<td>9.5%</td>
</tr>
<tr>
<td>Normal</td>
<td>4.93 0.39</td>
<td>7.9%</td>
</tr>
<tr>
<td>High</td>
<td>10 8.99 0.54</td>
<td>6.0%</td>
</tr>
</tbody>
</table>

*As measured in ten experiments in duplicate over a ten day period.

**14.2 Sensitivity**
The uE3 AccuBind® ELISA Test System has a sensitivity of 2.9 pg/ml. This is equivalent to a sample containing a concentration of 0.115 mg/ml. The sensitivity was ascertained by determining the variability of the 0 ng/ml serum calibrator and using the 2n (95% certainty) statistic to calculate the minimum dose.

**14.3 Accuracy**
The uE3 AccuBind® ELISA Test System was compared with a reference method. Biological specimens from low, normal and high Unconjugated Estriol level populations were used; the values ranged from 10.15 - 29.1 ng/ml. The total number of specimens was 158. The least square regression equation and the correlation coefficient were computed for this uE3 in comparison with this method. The data obtained is displayed in Table 5.

**TABLE 5**

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean (x)</th>
<th>Least Square Regression Analysis</th>
<th>Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Only slight amounts of bias between this method and the reference method are indicated by the closeness of the mean values. The least square regression equation and correlation coefficient indicates excellent method agreement.

**14.4 Specificity**
The % cross-reactivity of the Estriol antibody to selected substances, for determination of Unconjugated Estriol, was evaluated by adding the interfering substance to a serum pool containing massive concentrations. The cross-reactivity was calculated by deriving a ratio between dose of interfering substance to dose of uE3 needed to displace the same amount of labeled analog.

**15.0 REFERENCES**