1.0 INTRODUCTION

Intended Use: The Quantitative Determination of Unconjugated (Free) Estriol Concentration in Human Serum or Plasma by a Microplate Enzyme Immunoassay

2.0 SUMMARY AND EXPLANATION OF THE TEST

The last few years have seen the development of screening for fetal Down Syndrome by measurement of multiple markers in maternal blood. Although amniocentesis is widely used in the fetus, the plaques. The clinical evidence shows that in uncomplicated pregnancies, the production of estriol increases steadily throughout the pregnancy. Unconjugated estriol in the serum of pregnant women originates almost exclusively from precursors in the fetus, via the placenta. Changes in renal clearance and diurnal variations can make the results of these determinations suspect. In recent years investigators have found the determinations of unconjugated estriol in pregnancy plasma as an alternative to the urinary assay, to be a better marker of fetal stress. Abnormally low levels of estriol in a pregnant woman may indicate a problem with the development of the child. Levels of estriol in non-pregnant women do not change much after menopause, and levels are not significantly different from levels in men.

The Monobind unconjugated estriol EIA Kit uses a specific anti-estriol antibody, and does not require prior sample extraction of serum or plasma. Cross-reactivity to other naturally occurring and structurally related steroids is low.

The employment of several serum references of known Estriol concentration is used to establish the graph calibrating curve and concentration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with Estriol concentration.

3.0 PRINCIPLE

Competitive Enzyme Immunoassay (TYPE 7):

The essential reagents required for an enzyme immunoassay include antibody, enzyme-antigen conjugate and native antigen. Upon mixing biotinylated antibody, enzyme-antigen conjugate and a serum containing the native antigen, a competitive reaction results between the native antigen and the enzyme-antigen conjugate for a limited number of antibody binding sites. The interaction is illustrated by the following equation:

\[
A_{\text{Ag}} + A_{\text{AbBtn}} = K_a A_{\text{AgAbBtn}} + A_{\text{AgAbBtn}}
\]

\[
A_{\text{Ag}} = \text{Native Antigen (Variable Quantity)}
\]

\[
A_{\text{AbBtn}} = \text{Biotinylated Antibody (Constant Quantity)}
\]

\[
A_{\text{AgAbBtn}} = \text{Antigen-antibody Complex}
\]

\[
K_a = \text{Rate Constant of Association}
\]

\[
k_a = \text{Rate Constant of Dissociation}
\]

\[
K = k_a / k = \text{Equilibrium Constant}
\]

A simultaneous reaction between the biotin attached to the antibody and the streptavidin immobilized on the microwell occurs. This effects the separation of the antibody bound fraction after decantation or aspiration.

\[
A_{\text{Ag}} + A_{\text{EnzAgAbBtn}} = K_a A_{\text{AgEnzAgAbBtn}} + A_{\text{AgEnzAgAbBtn}}
\]

\[
A_{\text{EnzAg}} = \text{Enzyme-antigen Conjugate - Antibody Complex}
\]

\[
k = \text{Rate of Disassociation}
\]

\[
\text{Unconjugated Estriol (u-E3)}
\]

\[\text{Unconjugated estriol in the serum of pregnant women originates almost exclusively from precursors in the fetus, via the placenta. Changes in renal clearance and diurnal variations can make the results of these determinations suspect. In recent years investigators have found the determinations of unconjugated estriol in pregnancy plasma as an alternative to the urinary assay, to be a better marker of fetal stress. Abnormally low levels of estriol in a pregnant woman may indicate a problem with the development of the child. Levels of estriol in non-pregnant women do not change much after menopause, and levels are not significantly different from levels in men.}\]

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. Multiply the result by the dilution factor of 2 or 5 as required to obtain the concentration of the sample.

10.0 CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of unconjugated estriol in unknown specimens.

1. Record the absorbance obtained from the printout of the microplate reader; a minimum of two readings is recommended.

2. Plot the absorbance for each duplicate serum reference versus the corresponding unconjugated estriol concentration in ng/ml on linear graph paper (do not average the duplicates of the serum references before plotting).

3. Connect the obtained points; the obtained curve describes the dose response curve at 4.7 ng/ml unconjugated estriol concentration (See Figure 1).

Note: Computer data reduction software designed for ELISA assays may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

1. Format the microplates' wells for each calibrator, control and patient specimen to be assayed in duplicate. Place any unconjugated estriol assays back into the aluminum bag, seal and store at 2-8°C.

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

3. Add 0.050 ml (50µl) of the U-Estriol Enzyme Reagent to all wells (see Reagent Preparation Section).

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

5. Add 0.050 ml (50µl) of the U-Estriol Biotin Reagent to all wells.

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

7. Cover and incubate for 60 minutes at 2-8°C.

8. Discard the contents of the microplate by decapitation or aspiration; do not blot the plate dry with an absorbent paper.

9. Add 350µl of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes.

10. Add 0.100 ml (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.

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

12. Add 0.050ml (50µl) of stop solution to each well and gently mix for fifteen (15) seconds.

13. Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm).

14. Subtract blank value from each sample. Plot the results on graph paper as the absorbance at 450nm versus the corresponding unconjugated estriol concentration in ng/ml on linear graph paper.
TABLE 3
Within Assay Precision (Values in ng/ml)

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>X</th>
<th>σ</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>24</td>
<td>1.58</td>
<td>0.13</td>
<td>8.3%</td>
</tr>
<tr>
<td>Normal</td>
<td>24</td>
<td>2.17</td>
<td>0.37</td>
<td>7.1%</td>
</tr>
<tr>
<td>High</td>
<td>24</td>
<td>9.06</td>
<td>0.56</td>
<td>6.5%</td>
</tr>
</tbody>
</table>

TABLE 4
Between Assay Precision (Values in ng/ml)

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>X</th>
<th>σ</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>10</td>
<td>1.47</td>
<td>0.14</td>
<td>9.5%</td>
</tr>
<tr>
<td>Normal</td>
<td>10</td>
<td>4.93</td>
<td>0.39</td>
<td>7.9%</td>
</tr>
<tr>
<td>High</td>
<td>10</td>
<td>8.99</td>
<td>0.54</td>
<td>8.0%</td>
</tr>
</tbody>
</table>

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

11.0 Q.C. PARAMETERS

In order for the assay results to be considered valid the following criteria should be met:

1. The absorbance (OD) of calibrator 0 ng/ml should be > 1.3.
2. Four out of six quality control pools should be within the established ranges.

12.0 RISK ANALYSIS

The MSDS and Risk Analysis Form for this product is available on request from Monobind Inc.

12.1 Assay Performance

1. It is important that the time of reaction in each well is held constant to achieve reproducible results.
2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.
3. Highly lipemic, hemolyzed or grossly contaminated specimen(s) should not be used.
4. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
5. The addition of substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the substrate and stop solution should be added in the same sequence to eliminate any time-deviation during reaction.
6. Plate readers measure vertically. Do not touch the bottom of the wells.

14.4 Specificity

The % cross reactivity of the Estradiol antibody to selected substances was determined by dividing the sample matrix at massive concentrations. The cross-reactivity was calculated by deriving a ratio between dose of interfering substance to dose of Unconjugated Estradiol needed to displace the same amount of labeled analog.

It is important to keep in mind that establishment of a range of values which can be expected to be found by a given method for a population of "normal" persons is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons each laboratory should depend upon the range of expected values established by the manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

14.0 PERFORMANCE CHARACTERISTICS

14.1 Precision

The within and between assay precision of the Unconjugated Estradiol AccuBind™ Microplate EIA Test System were determined by analyses on 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.

15.0 REFERENCES