Monobind Inc.
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AccuBind
ELISA Microwells

Unconjugated Estriol (u-E3) Test System
Product Code: 5025-300

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 [1]. Although amniocentesis has been widely available for more than 40 years it can only be selectively used to diagnose the disorder because of the hazard to fetus. Of most employed for differential diagnosis the commonly used procedures are AFP, hCG, free beta-HCG and unconjugated estriol.[2]

Unconjugated estriol in the serum of pregnant women originates almost exclusively from precursors in the fetus, via the placenta.[3] The clinical evidence shows that in uncomplicated pregnancies, the production of estriol increases steadily throughout the last trimester; however, in pregnancies complicated by placental insufficiency the synthesis of estriol decreases rapidly. For many years the most commonly used method for monitoring estriol synthesis (as an index to fetal stress) has been to measure estriol and estriol conjugates in a 24 hr urine sample[4]. However, 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[6]. 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 [7].

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 permits construction of a graph of activity 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 a 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 competition 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:

$$^{Enz}Ag + Ag + Ab_{Btn} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} AgAb_{Btn} + {}^{Enz}AgAb_{Btn}$$

Ab_{Btn} = Biotinylated Antibody (Constant Quantity)
Ag = Native Antigen (Variable Quantity)
^{Enz}Ag = Enzyme-antigen Conjugate (Constant Quantity)
$AgAb_{Btn}$ = Antigen-Antibody Complex
$^{Enz}Ag\ Ab_{Btn}$ = Enzyme-antigen Conjugate -Antibody Complex
k_a = Rate Constant of Association
k_{-a} = Rate Constant of Disassociation
$K = k_a / k_{-a}$ = 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.

$AgAb_{Btn} + {}^{Enz}AgAb_{Btn} + \underline{Streptavidin}_{CW} \Rightarrow$ <u>immobilized complex</u>

$\underline{Streptavidin}_{CW}$ = Streptavidin immobilized on well

<u>Immobilized complex</u> = sandwich complex bound to the solid surface

The enzyme activity in the antibody bound fraction is inversely proportional to the native antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

4.0 REAGENTS

A. u-Estriol Calibrators – 1ml/vial - Icons A-F
Six (6) vials of serum reference for unconjugated estriol at concentrations of 0 (**A**), 0.4 (**B**), 2.0 (**C**), 5.0 (**D**), 15 (**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 (nM/L) by the conversion factor 3.45. For example: 1ng/ml x 3.45 = 3.45 nM/L

B. u-Estriol Enzyme Reagent – 6.0 ml/vial
One (1) vial of Estriol (Analog)-horseradish peroxides (HRP) conjugate in a protein stabilizing matrix with red dye. Store at 2-8°C.

C. u-Estriol Biotin Reagent – 6.0 ml - Icon
One (1) bottle of reagent 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 96-well microplate coated with 1.0 µg/ml streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

E. Wash Solution Concentrate – 20ml - Icon
One (1) vial contains a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

F. Substrate A – 7ml/vial - Icon S^A
One (1) vial contains tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.

G. Substrate B – 7ml/vial - Icon S^B
One (1) vial contains hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

H. Stop Solution – 8ml/vial - Icon
One (1) vial contains a strong acid (1N HCl). Store at 2-30°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 25 µl and 50 µl with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100ml and 0.350ml 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 Absorbent Paper for blotting the microplate wells.
7 Plastic wrap or microplate cover for incubation steps.
8 Vacuum aspirator (optional) for wash steps.
9 Timer.
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 HCV 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, "Biosafety in Microbiological and Biomedical Laboratories," 2nd Edition, 1988, 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 heparanised plasma in type and taken with the usual precautions in the collection of venipuncture samples. The blood should be collected in a redtop (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.

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. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050ml of the specimen is required.

7.0 QUALITY CONTROL

Each laboratory should assay controls at levels in the low, normal and high range for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. The individual laboratory should set acceptable assay performance limits. In addition, maximum absorbance should be consistent with past experience. Significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

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**
   Pour the contents of the amber vial labeled Solution 'A' into the clear vial labeled Solution 'B'. Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. Store at 2 - 8°C.

Note1 : Do not use the working substrate if it looks blue.
Note 2: Do not use reagents that are contaminated or have bacteria growth.

9.0 TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum references 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 in duplicate. **Replace any unused microwell strips 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 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 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 room temperature.
8. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with 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. **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 0.100 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.**
    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 620-630nm. **The results should be read within thirty (30) minutes of adding the 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. 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.

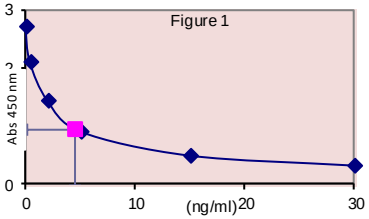
1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the absorbance for each duplicate serum reference versus the corresponding unconjugated estriol concentration in ng/ml on linear graph paper (do not average the duplicates of the serum references before plotting).
3. Connect the points with a best-fit curve.
4. 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 example, the average absorbance (0.967) intersects the dose response curve at (4.71 ng/ml) unconjugated estriol concentration (See Figure 1).

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

EXAMPLE 1

Sample I.D.	Well Number	Abs (A)	Mean Abs (B)	Value (ng/ml)
Cal A	A1	2.742	2.732	0
	B1	2.722		
Cal B	C1	2.155	2.123	0.4
	D1	2.091		
Cal C	E1	1.492	1.456	2.0
	F1	1.420		
Cal D	G1	0.940	0.921	5.0
	H1	0.903		
Cal E	A2	0.523	0.508	15.0
	B2	0.493		
Cal F	C2	0.342	0.336	30.0
	D2	0.330		
Ctrl 1	G2	1.557	1.532	***1.82***
	H2	1.507		
Patient	A3	0.991	0.967	***4.71***
	B3	0.943		

*The data presented in Example 1 and Figure 1 is for illustration only and **should not** be used in lieu of a standard curve prepared with each assay.



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.
7. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
8. Use components from the same lot. No intermixing of reagents from different batches.
9. Patient specimens with Unconjugated E3 concentrations above 30 ng/ml may be diluted (1/2,1/5 or higher) with Unconjugated E3 '0' calibrator and re-assayed. The sample's concentration is obtained by multiplying the result by the dilution factor.
10. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from Monobind's IFU may yield inaccurate results.
11. All applicable national standards, regulations and laws, including, but not limited to, good laboratory procedures, must be strictly followed to ensure compliance and proper device usage.
12. It is important to calibrate all the equipment e.g. Pipettes, Readers, Washers and/or the automated instruments used with this device, and to perform routine preventative maintenance.
13. Risk Analysis- as required by CE Mark IVD Directive 98/79/EC - for this and other devices, made by Monobind, can be requested via email from Monobind@monobind.com.

12.2 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 not be the sole basis for therapy, particularly if the results conflict with other determinants.
3. 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.
5. 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.

13.0 EXPECTED RANGES OF VALUES

In agreement with established reference intervals[5] for a "normal" adult population, the expected ranges for the Unconjugated Estriol AccuBind™ ELISA Test System are detailed in Table 1.

TABLE I
Expected Values for the Unconjugated Estriol EIA Test System (ng/ml)

Male & Non-Pregnant Female	**< 1.0 ng/ml**

During pregnancy the Unconjugated E3 serum levels rise rapidly till the end of third trimester. (See Table 2 from published Literature).[6]

TABLE 2

Gestation Week	Expected Range (ng/ml)	Gestation Week	Expected Range (ng/ml)	Twin Pregnancy (ng/ml)
12	0.3 – 1.0	22	2.7 – 16.0	3.0 -18.0
14	0.4 – 1.6	26	3.0 – 18.0	4.0 – 21.0
16	1.4 – 6.5	32	4.6 – 23.0	5.0 – 25.0
18	1.6 – 8.5	36	7.2 – 29.0	7.0 – 39.0
20	2.1 – 13.0	40	8.0 – 39.0	13.0 – 40.0

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 Estriol AccuBind™ Microplate EIA 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
Within Assay Precision (Values in ng/ml)

Sample	N	X	σ	C.V.
Low	24	1.58	0.13	8.3%
Normal	24	5.17	0.37	7.1%
High	24	9.06	0.59	6.5%

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

Sample	N	X	σ	C.V.
Low	10	1.47	0.14	9.5%
Normal	10	4.93	0.39	7.9%
High	10	8.99	0.54	6.0%

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

14.2 Sensitivity

The Unconjugated Estriol AccuBind™ Microplate EIA Test System has a sensitivity of 2.9 pg/T. This is equivalent to a sample containing a concentration of 0.115 ng/ml. The sensitivity was ascertained by determining the variability of the 0 ng/ml serum calibrator and using the 2σ (95% certainty) statistic to calculate the minimum dose.

14.3 Accuracy

The unconjugated Estriol AccuBind™ Microplate EIA Test System was compared with a chemiluminescence immunoassay method. Biological specimens from low, normal and high Unconjugated Estriol level populations were used; the values ranged from 0.15 – 29.1 ng/ml. The total number of specimens was 158. The least square regression equation and the correlation coefficient were computed for this unconjugated Estriol EIA in comparison with the reference method. The data obtained is displayed in Table 5.

TABLE 5

Method	Mean (x)	Least Square Regression Analysis	Correlation Coefficient
This Method (y)	3.84	y= -0.174+0.979(x)	0.952
Reference (X)	3.74		

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 matrix at massive concentrations. The cross-reactivity was calculated by deriving a ratio between dose of interfering substance to dose of Unconjugated Estriol needed to displace the same amount of labeled analog.

Substance	% Cross Reactivity
Estriol	100.0000
Androstenedione	0.0001
Cortisol	<0.0001
Cortisone	<0.0001
Corticosterone	<0.0001
DHEA-S	<0.0001
Dihydotestosterone	0.0001
Estradiol	0.0040
Estriol Glucouronide	<0.0001
Estriol Sulfate	0.6200
Estrone	0.0004
Prednisone	<0.0001
Progesterone	<0.0001
Spirolactone	<0.0001
Testosterone	<0.0001

15.0 REFERENCES

1. Goebelsman, U, Katagiri, H, Stanczyk et al., Estriol assays in obstetrics. *J. Steroid Biochemistry.* 6, 703-709 (1975).
2. Reynolds T, Penny M, "The mathematical basis of multivariate risk screening, with special reference to screening for Down Syndrome associated pregnancy", *Ann Clin Biochem* , **27**:452-458 (1990).
3. Kastagiri,H., Stanczyk, F and Goebelsman,U, "Estriol in pregnancy.III Development, comparison and use of a specific antisera for rapid radioimmunoassay of unconjugated estriol in pregnancy plasma", *Steroids,* **24**, 225 (1974).
4. Brown CH, Saffan, BD, Howard C.M and Preedy JR. "The renal clearance of endogenous estrogens in late pregnancy", *J Clinical Investigation,* .**43**,295 (1964).
5. Tietz, NW, ED: Clinical Guide to Laboratory Tests, 3rd ed. Philadelphia, WA Saunders Co, 1995.
6. Cohen M, and Cohen H, "A radioimmunoassay for plasma unconjugated estrogens in normal pregnancy", *Am J. Obstet. Gynecology,* **118,** 200 (1974).
7. Wright JV, Schliesman B, Robinson L, "Comparative measurements of serum estriol, estradiol and estrone in non-pregnant, postmenopausal women; a preliminary investigation:, *Altern Med Rev* , **4** 266-70 (1999).
8. NIH State-of-the-Science Conference Statement on Management of Menopause-Related Symptoms. NIH Consensus State Sci Statements. 2005. Mar 21-23; 22(1) 1–38.

Rev 3 **Date: 030912** **DCO: 0638**
Cat #: 5025-*300*

Size		96(A)	192(B)
Reagent (fill)	A)	1ml set	1ml set
	B)	1 (6ml)	2 (6ml)
	C)	1 (6ml)	2 (6ml)
	D)	1 plate	2 plates
	E)	1 (20ml)	1 (20ml)
	F)	1 (7ml)	2 (7ml)
	G)	1 (7ml)	2 (7ml)
	H)	1 (8ml)	2 (8ml)

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