Monobind Inc.
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AcuLite
CLIA Microwells

Unconjugated Estriol (uE3) Test System
Product Code: 5075-300

1.0 INTRODUCTION

Intended Use: The Quantitative Determination of Free (unconjugated) Estriol (uE3) Concentration in Human Serum or Plasma by a Microplate Enzyme Immunoassay, Chemiluminescence

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 for 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 in 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 CLIA 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 (light) 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 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 followed equation:

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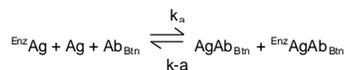
Ab_{Btn} = Biotinylated Antibody (Constant Quantity)
Ag = Native Antigen (Variable Quantity)
^{Enz}Ag = Enzyme-antigen Conjugate (Constant Quantity)
$AgAb_{Btn}$ = Antigen-Antibody Complex
$^{Enz}AgAb_{Btn}$ = Enzyme-antigen Conjugate -Antibody Complex
k_a = Rate Constant of Association
k_{-a} = Rate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

$AgAb_{Btn} + {}^{Enz}AgAb_{Btn} + Streptavidin_{CW} \Rightarrow$ immobilized complex
$Streptavidin_{CW}$ = Streptavidin immobilized on well
Immobilized complex = 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

Materials Provided:

A. uE3 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) using the conversion factor 3.45. For example: 1ng/ml x 3.45 = 3.45 nM/L.

B. uE2 Tracer Reagent – 6.0 ml/vial – Icon E
One (1) vial contains Estriol (Analog)-horseradish peroxides (HRP) conjugate 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. Light Reaction Wells – 96 wells – Icon ⇓
One 96-well white microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

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

F. Signal Reagent A – 7.0ml/vial - Icon C^A
One (1) vial contains luminol in a buffer. Store at 2-8°C.

G. Signal Reagent B – 7.0ml/vial - Icon C^B
One (1) vial contains hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

H. Product Insert.

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 Luminometer.
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 requirements.

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.

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 2-8ºC 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 -20ºC for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050ml (50μl) 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 Concentrate to 1000ml with distilled or deionized water in a suitable storage container. Store diluted buffer 2-30°C for up to 60 days.
2. **Working Signal Reagent Solution -** Store at 2 - 30°C. Determine the amount of reagent needed and prepare by mixing equal portions of Signal Reagent A and Signal Reagent B in a clean container. For example, add 1 ml of A and 1 ml of B per two (2) eight well strips (A slight excess of solution is made). **Discard the unused portion if not used within 36 hours after mixing.** If complete utilization of the reagents is anticipated, within the above time constraint, pour the contents of Signal Reagent B into Signal Reagent A and label accordingly.

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℃).
Test procedure should be performed by a skilled individual or trained professional

1. Format the microplates' wells for each serum reference, 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 calibrator, control or specimen into the assigned well.
3. Add 0.050 ml (50μl) of the u-Estriol Tracer Reagent to all wells.
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 45 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 (tap and blot) or aspirate. Repeat four (4) additional times for a total of five (5) 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 four (4) additional times.**
10. Add 0.100 ml (100μl) of working signal reagent solution to all wells (see Reagent Preparation Section). **Always add reagents in the same order to minimize reaction time differences between wells.**
11. **DO NOT SHAKE THE PLATE AFTER SiGNAL ADDITION**
12. Incubate at room temperature for five (5) minutes in the dark.
13. Read the relative light units in each well with a Chemiluminescence microplate reader for 0.5-1.0 seconds. **The results should be read within 30 minutes after adding the working Signal Reagent.**

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 estriol in unknown specimens.
1. Record the RLUs obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the RLUs for each duplicate serum reference versus the corresponding Progesterone concentration in ng/ml on linear graph paper.
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of estriol for an unknown, locate the average RLUs 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 RLUs (31308) of the unknown intersects the calibration curve at (5.08) estriol concentration (see figure 1).

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

EXAMPLE 1

Sample I.D.	Well Number	RLU (A)	Mean RLU (B)	Value (ng/ml)
Cal A	A1	99755	100000	0
	B1	100245		
Cal B	C1	76214	76295	0.4
	D1	76377		
Cal C	E1	51445	51662	2.0
	F1	51879		
Cal D	G1	31093	32063	5.0
	H1	33033		
Cal E	A2	13582	13831	15.0
	B2	14080		
Cal F	C2	8061	8069	30.0
	D2	8077		
Ctrl 1	G2	57568	57510	*1.42*
	H2	57452		
Patient	A3	30186	31308	*5.08*
	B3	32430		
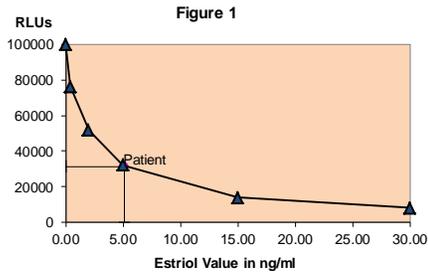
Figure 1

* The data presented in Example 1 and Figure 1 is for illustration only and **should not** be used in lieu of a dose response curve prepared with each assay. In addition, the RLUs of the calibrators have been normalized to 100,000 RLUs for the A calibrator (greatest light output). This conversion minimizes differences caused by efficiency of the various instruments that can be used to measure light output.

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 $\geq$ 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 are 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 signal reagent initiates a kinetic reaction, therefore the signal reagent(s) should be added in the same sequence to eliminate any time-deviation during reaction.
6. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
7. Use components from the same lot. No intermixing of reagents from different batches.
8. 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.

9. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from Monobind IFU may yield inaccurate results.
10. 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.
11. 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.
12. 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. 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. Heterophilic antibodies often cause these interactions and have been known to be problems for all kinds of immunoassays (Boscato LM, Stuart MC. 'Heterophilic antibodies: a problem for all 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.
4. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
5. 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.
6. 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 AccuLite® CLIA Test System are detailed in Table 1.

TABLE I
Expected Values for the uEstriol CLIA 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 AccuLite® CLIA 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	20	1.71	0.07	4.5%
Normal	20	5.79	0.32	5.6%
High	20	11.17	0.37	3.3%

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

Sample	N	X	σ	C.V.
Low	20	1.68	0.15	9.1%
Normal	20	6.88	0.52	7.5%
High	20	11.93	0.67	5.6%

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

14.2 Sensitivity
The Unconjugated Estriol AccuLite® CLIA Test System has a sensitivity of 0.006 ng/well. This is equivalent to a sample containing a concentration of 0.237 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 AccuLite® CLIA 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 CLIA 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
Monobind (y)	4.30	Y=-0.071+0.880(X)	0.991
Reference(x)	4.96		

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).
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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).
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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)

Glossary of Symbols
(EN 980/ISO 15223)

IVD In Vitro - Diagnostic Medical Device	Temperature Limitation Storage Condition (2-8° C)	Consult Instructions for Use
REF Catalogue Number	Σ Contains Sufficient Test for Σ	**LOT** Batch Code
Used By (Expiration Day)	Date of Manufacturer	Manufacturer
EC REP Authorized Rep in European Country	**CE** European Conformity	