Monobind Inc.
Lake Forest, CA 92630, USA

AccuLite
CLIA Microwells

17α-OH Progesterone (17-OHP) Test System

Product Code: 5275-300

1.0 INTRODUCTION

Intended Use: The Quantitative Determination of 17α-OH Progesterone Concentration in Human Serum or Plasma by a Microplate Enzyme Immunoassay, Chemiluminescence

2.0 SUMMARY AND EXPLANATION OF THE TEST

Plasma/Serum concentrations of 17α-hydroxyprogesterone (17α-OHP) are valuable in the initial diagnosis of congenital adrenal hyperplasia (CAH).[1, 2] This common inborn error of metabolism is usually characterized by deficiency in the C21-hydroxylase enzyme system, and necessitates steroid replacement therapy. Adequacy of treatment has been monitored by determining circulating 17α-OHP concentrations.[3, 4]

The incidence is roughly estimated to be 1 in 15,000 newborns and can reach as high as 1 in 1480 in native Alaskans. Early diagnosis is valuable to detect CAH in newborns afflicted with the disease, not clinically recognizable, but which will lead to life threatening adrenal crisis in the neonatal period and to determine the cause of infants with ambiguous genitalia. Delayed diagnosis may also lead to further virilization in female children, acceleration of skeletal maturation and premature development of secondary sex characteristics in male children. Prompt treatment can save the life of infants and allow afflicted children to attain normal growth.

17P is a steroid produced in the adrenal cortex and the gonads. It is the immediate precursor to 11-desoxycortisol (CpS) which is converted to cortisol. Because CpS is produced by 21-hydroxylation of 17P, measurement of 17P is an indirect indicator of 21-hydroxylase activity. CAH occurs where there is a deficiency of this enzyme. The result is a decrease in the conversion of 17P to CpS which blocks the normal synthesis of cortisol. Due to the feed back mechanism, a decrease in cortisol causes an increase in ACTH secretion resulting in adrenal hyperplasia. As 17P is not being converted, increased concentrations of this steroid will be found.

17P concentration increases during pregnancy in the maternal and fetal blood. After birth, values decline rapidly to reach normal adult values in 2 to 7 days. Thus it is advisable not to collect samples before the 3rd day of life. Premature and sick term infants exhibit 2 to 3 fold 17P values with no CAH disorder. It is suggested that a different cut off be adapted to pre-term and sick infants.

In this method, a sample containing 17-OH progesterone is dispensed into a microplate well. An enzyme labeled 17OH progesterone derivative and biotinylated anti-17OH-progesterone are than added. After a suitable incubation, the antibody fraction is separated from unbound enzyme reagent.

The employment of several serum references of known 17α-OH Progesterone 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 17α-OH Progesterone 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:

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

Ab_{Btn} = Biotinylated Antibody (Constant Quantity)
Ag = Native Antigen (Variable Quantity)
^{Enz}Ag = Enzyme-antigen Conjugate (Constant Quantity)
$AgAb_{Btn}$ = Antigen-Antibody Complex
$^{Enz}AgAb_{Btn}$ = 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.

$AgAb_{Btn} + {}^{Enz}AgAb_{Btn} + \underline{Streptavidin}_{CW} \Rightarrow \underline{immobilized\ complex}$
$\underline{Streptavidin}_{CW}$ = 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. 17-OHP Calibrators – 1ml/vial – Icons A-F
Six (6) vials of serum reference for 17α-OH Progesterone at concentrations of 0 (**A**), 0.1 (**B**), 0.5 (**C**), 1.0 (**D**), 2.5 (**E**), and 10 (F) ng/ml. Store at 2-8°C. A preservative has been added. The calibrators can be expressed in molar concentrations (nM/L) by multiplying by 3.03.
For example: 1ng/ml x 3.03 = 3.03 nM/L

B. 17-OHP Tracer Reagent – 6 ml/vial – Icon Ⓔ
One (1) ready to use vial containing 17-OH Progesterone (Analog)-horseradish peroxides (HRP) conjugate in a protein stabilizing matrix with buffer, preservative, binding protein inhibitors, and dye. Store at 2-8°C.

C. 17-OHP Biotin Reagent – 6 ml/vial - Icon ∇
One (1) vial containing anti-17α-OH Progesterone 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 Concentrate – 20ml/vial – Icon
One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

F. Signal Reagent A – 7 ml/vial – Icon C[A]
One (1) vial containing luminol in a buffer. Store at 2-8°C.

G. Signal Reagent B – 7 ml/vial – Icon C[B]
One (1) vial containing hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

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

4.1 Required But Not Provided:

1. Pipette capable of delivering 0.025 & 0.050 ml (25 & 50 µl) with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.050ml (50µl), 0.100 (100µl) & 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 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. For accurate comparison to establish normal values, a fasting morning serum sample should be obtained. 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 2-8°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, 0.050 ml (50 µl) 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 2-30°C for up to 60 days.
2. **Working Signal Reagent Solution -** Store at 2 - 8°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 1: 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 serum reference 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 (25 µl) of the appropriate serum reference, control or specimen into the assigned well.
3. Add 0.050 ml (50 µl) of ready to use 17-OHP Tracer Reagent to all wells.
4. Swirl the microplate gently for 20-30 seconds to mix.
5. Add 0.050 ml (50 µl) of the 17-OHP 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 0.350 ml (350 µl) 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 (100 µl) 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. Incubate at room temperature for five (5) minutes in the dark.
12. Read the relative light units (RLUs) 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 samples suspected of concentrations higher than 10ng/ml 1:1 and 1:5 with 17α-OH Progesterone '0' ng/ml calibrator or male patient serum pools with a known low value for 17-OH Progesterone.

10.0 CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of 17α-OH Progesterone in unknown specimens.

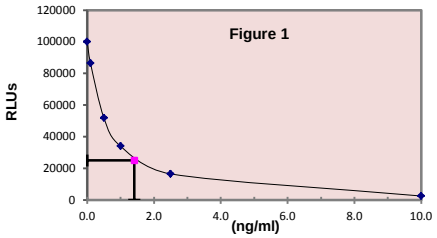
1. Record the RLUs obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the RLUs for each duplicate serum reference versus the corresponding 17-OHP concentration in ng/ml on linear graph paper (do not average the duplicates of the serum references before plotting).
3. Connect the points with a best-fit curve.
4. To determine the concentration of 17-OHP for an unknown, locate the average RLUs 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 RLUs (24948) intersects the dose response curve at 1.51 ng/ml 17-OHP concentration (See Figure 1).

Note: Computer data reduction software designed for chemisluminescence 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	RLUs (A)	Mean RLU (B)	Value (ng/ml)
Cal A	A1	102417	100000	0
	B1	97583		
Cal B	C1	87931	86389	0.1
	D1	84847		
Cal C	E1	52738	51901	0.5
	F1	51084		
Cal D	G1	34875	33964	1.0
	H1	33253		
Cal E	A2	16975	16450	2.5
	B2	15924		
Cal F	C2	2608	2474	10
	D2	2339		
Pat# 1	A3	24653	24948	1.51
	B3	25243		

***** 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. Do not use for calculating results. 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. 1.The Dose Response Curve should be within established parameters.
2. 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 upon 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. 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.
9. 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.
10. 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.
11. 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 used 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 for a "normal" adult population and females during gestation the expected ranges for the 17-OHP AccuLite® CLIA Test System are detailed in Table 1.

TABLE I
Expected Values for the 17-OHP Test System

	(ng/ml)	(nmol/L)
Prepubertal Child (1-10 yr)	0.2 – 0.8	0.64 – 2.54
Adult man	0.2 – 3.1	0.64 – 9.86
Adult woman		
Follicular phase	0.20-1.30	0.64 – 4.13
Luteal phase	1.00 – 4.51	3.18 – 14.34
Postmenopausal woman	0.2 – 0.9	0.64 – 2.86

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 17-OHP 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 2 and Table 3.

TABLE 2
Within Assay Precision (Values in ng/ml)

Sample	N	X	σ	C.V.
Low	20	1.0	0.08	8.0%
Normal	20	3.5	0.21	6.0%
High	20	7.9	0.40	5.1%

TABLE 3
Between Assay Precision (Values in ng/ml)

Sample	N	X	σ	C.V.
Low	10	1.1	0.10	9.1%
Normal	10	3.6	0.23	6.4%
High	10	7.5	0.38	5.1%

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

14.2 Sensitivity

The 17-OHP AccuLite® CLIA Test System has a sensitivity of 0.040 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 17-OHP AccuLite® CLIA Test System was compared with a chemiluminescence immunoassay method. Biological specimens from low, normal and high 17-OH Progesterone level populations were used. (The values ranged from < 0.15 ng/ml – 128 ng/ml.) The total number of such specimens was 66. The least square regression equation and the correlation coefficient were computed for this method in comparison with the reference method. The data obtained is displayed in Table 4.

TABLE 4

Method	Mean (x)	Least Square Regression Analysis	Correlation Coefficient
Monobind (Y)	3.19	Y= 0.2232+0.935(x)	0.932
Reference (X)	3.38		

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 17α-OH Progesterone antibody to selected substances was evaluated by adding the interfering substance to a serum matrix at various concentrations. The cross-reactivity was calculated by deriving a ratio between dose of interfering substance to dose of 17-OH Progesterone needed to displace the same amount of labeled analog.

Substance	Cross Reactivity
17α-OH Progesterone	100.000
Progesterone	0.375
Androstenedione	0.158
Cortisone	0.014
Corticosterone	0.347
Cortisol	0.005
Danazol	0.003
Dihydotestosterone	0.006
DHEA sulfate	0.002
Estradiol	0.004
Estrone	0.003
Estriol	0.002
Prednisone	0.023
Testosterone	0.015
RF	<0.001

15.0 REFERENCES

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MP5275 **Product Code: 5275-300**

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)

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IVD 2° 8° CE
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Please visit our website to learn more about our products and services.

Glossary of Symbols
(EN 980/ISO 15223)

IVD — In Vitro - Diagnostic Medical Device
2° 8° — 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