Monobind Inc.
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AccuBind
ELISA Microwells

β-Human Chorionic Gonadotropin

Product Code: 825-300

Intended Use: The Quantitative Determination of Chorionic Gonadotropin (hCG) Concentration in Human Serum by a Microplate Immunoenzymometric assay

SUMMARY AND EXPLANATION OF THE TEST

Human chorionic gonadotropin (hCG) concentration increases dramatically in blood and urine during normal pregnancy. hCG is secreted by placental tissue, beginning with the primitive trophoblast, almost from the time of implantation, and serves to support the corpus luteum during the early weeks of pregnancy. hCG or hCG similar glycoproteins can also be produced by a wide variety of trophoblastic and nontrophoblastic tumors. The measurement of hCG, by assay systems with suitable sensitivity and specificity has proven great value in the detection of pregnancy and the diagnosis *of early* pregnancy disorders.

According to the literature, hCG is detectable as early as 10 days after ovulation, reaching 100 mIU/ml by the first missed period. At the time for the next ovulation, the hCG level is 200 mIU/ml (approximately 28 days after conception) (1). A peak of 50,000 or even 100,000 mIU/ml is attained by the third month, then a gradual decline is observed (2, 3).

In this method, hCG calibrator, patient specimen or control is first added to a streptavidin coated well. Biotinylated monoclonal and enzyme labeled antibodies (directed against distinct and different epitopes of hCG) are added and the reactants mixed. Reaction between the various hCG antibodies and native hCG forms a sandwich complex that binds with the streptavidin coated to the well.

After the completion of the required incubation period, the enzyme-chorionic gonadotropin antibody bound conjugate is separated from the unbound enzyme-chorionic gonadotropin conjugate by aspiration or decantation. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce color.

The employment of several serum references of known chorionic gonadotropin levels permits construction of a dose response curve of activity and concentration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with chorionic gonadotropin concentration.

PRINCIPLE

Immunoenzymometric assay (TYPE 3):

The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme and immobilized), with different and distinct epitope recognition, **in excess,** and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-hCG antibody.
Upon mixing monoclonal biotinylated antibody, the enzyme-labeled antibody and a serum containing the native antigen, reaction results between the native antigen and the antibodies without competition or steric hindrance to form a soluble sandwich complex. The interaction is illustrated by the following equation:

$$^{Enz}Ab_{(x\text{-}hCG)} + Ag_{hCG} + {}^{Btn}Ab_{(m)} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} {}^{Enz}Ab_{(m)}\text{-}Ag_{hCG}\text{-}{}^{Btn}Ab_{(m)}$$

$^{Btn}Ab_{(m)}$ = Biotinylated Monoclonal Antibody (Excess Quantity)

Ag_{hCG} = Native Antigen (Variable Quantity)

$^{Enz}Ab_{(x\text{-}hCG)}$ = Enzyme labeled Antibody (Excess Quantity)

$^{Enz}Ab_{(hCG)}\text{-}Ag_{hCG}\text{-}{}^{Btn}Ab_{(m)}$ =Ag-Antibodies Sandwich complex

k_a = Rate Constant of Association

k_{-a} = Rate Constant of Dissociation

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:

$$^{Enz}Ab_{(x\text{-}hCG)}\text{-}Ag_{hCG}\text{-}{}^{Btn}Ab_{(m)} + Streptavidin_{CW} \Rightarrow \text{immobilized complex}$$

$Streptavidin_{CW}$ = Streptavidin immobolized on well
Immobilized complex = sandwich complex bound to the well

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

REAGENTS

A. hCG Calibrators --1 ml/vial - Icons A-F
Six (6) vials of references for hCG Antigen at levels of 0(**A**), 5(**B**), 25(**C**), 50(**D**), 100(**E**) and 250(**F**) mIU/ml. Store at 2-8°C. A preservative has been added.

Note: The calibrators, human serum based, were calibrated using a reference preparation, which was assayed against the WHO 3rd IS (75/537).

B. hCG Enzyme Reagent —13 ml/vial - Icon
One (1) vial containing enzyme labeled affinity purified antibody, biotinylated monoclonal mouse IgG in buffer, dye, and preservative. Store at 2-8°C.

C. Streptavidin Coated Plate-- 96 wells - Icon
One 96-well microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

D. Wash Solution – 20 ml - Icon
One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-30°C.

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

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

G. Stop Solution -- 8ml/vial - Icon (STOP)
One (1) bottle containing a strong acid (1N HCl). Store at 2-30°C.

H. Product Instructions.

Note 1: Do not use reagents beyond the kit expiration date.
Note 2: Opened reagents are stable for sixty (60) days when stored at 2-8°C.
Note 3: Above reagents are for a single 96-well microplate

Required But Not Provided:

1. Pipette(s) capable of delivering 25 & 50 volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100ml and 0.300ml volumes with a precision of better than 1.5%.
3. Microplate washers or a squeeze bottle (optional).
4. Microplate Reader with 450nm and 620nm wavelength absorbance capability.
5. Absorbent Paper for blotting the microplate wells.
6. Plastic wrap or microplate cover for incubation steps.
7. Vacuum aspirator (optional) for wash steps.
8. Timer.
9. Quality control materials

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 licensed reagents. 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.

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood, serum in type and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or anti-coagulants. Allow the blood to clot. Centrifuge the specimen to separate the serum 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 repetitive freezing and thawing. When assayed in duplicate, 0.05 ml of the specimen is required.

REAGENT PREPARATION:

1. **Wash Buffer**
   Dilute contents of wash solution to 1000ml with distilled or deionized water in a suitable storage container. Store at room temperature 20-27°C for up to 60 days.

2. **Working Substrate Solution**
   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.

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

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20-27°C).

1. Format the microplate 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 serum reference, control or specimen into the assigned well.
3. Add 0.100 ml (100µl) of hCG-Enzyme Reagent to all wells.
4. Swirl the microplate gently for 20-30 seconds to mix and cover.
5. Incubate 60 minutes at room temperature.
6. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.
7. Add 300µl 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.**
8. 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
9. Incubate at room temperature for fifteen (15) minutes.
10. 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**
11. Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader. **The results should be read within thirty (30) minutes of adding the stop solution.**

QUALITY CONTROL

Each laboratory should assay controls at levels in the low, normal and elevated 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. 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.

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of Human chorionic gonadotropin (hCG) 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 hCG concentration in mIU/ml on linear graph paper (do not average the duplicates of the serum references before plotting).
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of hCG 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 mIU/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 (1.745) intersects the dose response curve at (157 mIU/ml) hCG concentration (See Figure 1).

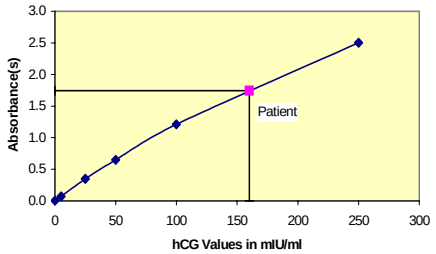
Note: Computer data reduction software designed for IEMA/ Elisa assays may also be used for the data reduction

EXAMPLE 1

Sample I.D.	Well Number	Abs (A)	Mean Abs (B)	Value (mIU/ml)
Cal A	A1	0.002	0.004	0
	B1	0.005		
Cal B	C1	0.073	0.071	5
	D1	0.069		
Cal C	E1	0.340	0.350	25
	F1	0.360		
Cal D	G1	0.637	0.650	50
	H1	0.663		
Cal E	A2	1.223	1.212	100
	B2	1.199		
Cal F	C2	2.518	2.502	250
	D2	2.486		
Ctrl 1	E2	0.075	0.076	**5.8**
	F2	0.077		
Ctrl 2	G2	0.280	0.290	**21.9**
	H2	0.301		
Patient	A3	1.736	1.745	**157**
	B3	1.754		

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

Figure 1



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 'F' should be $\geq$ 1.3.
2. Four out of six quality control pools should be within the established ranges.

LIMITATIONS OF PROCEDURE

A. Assay Performance

1. It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
2. Addition of the substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the addition of the substrate and the stopping solution should be added in the same sequence to eliminate any time deviation during reaction.
3. Plate readers measure vertically. Do not touch the bottom of the wells.
4. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
5. Sample(s), which are contaminated microbiologically, should not be used in the assay. Highly lipemeic or hemolysed specimen(s) should similarly not be used.
6. Patient specimens with hCG concentrations above 250 mIU/ml may be diluted with normal male serum (hCG < 1 mIU/ml) and re-assayed. The sample's concentration is obtained by multiplying the result by the dilution factor.
7. Each component in one assay should be of the same lot number and stored under identical conditions.

B. Interpretation

1. 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.
2. False positive results may occur in the presence of a wide variety of trophoblastic and nontrophoblastic tumors that secrete hCG. Therefore, the possibility of an hCG secreting neoplasia should be eliminated prior to diagnosing pregnancy.
3. Also, false positive results may be seen when assaying specimens from individuals taking the drugs Pergonal* and Clomid**. Additionally Pergonal will often be followed with an injection of hCG.
4. Spontaneous microabortions and ectopic pregnancies will tend to have values which are lower than expected during a normal pregnancy while somewhat higher values are often seen in multiple pregnancies (4, 5, 6).
5. Following therapeutic abortion, detectable hCG may persist for as long as three to four weeks. The disappearance rate of hCG, after spontaneous abortion, will vary depending upon the quantity of viable residual trophoblast (4, 5, 6, 7).

*Pegonal is a registered trademark of Serono Laboratories, Inc.
**Clomid is a registered trademark of Merriell-National Laboratories

EXPECTED RANGES OF VALUES

A study of an apparent normal adult population was undertaken to determine expected values for the HCG AccuBind™ ELISA Test System. The mean (X) values, standard deviations (σ) and expected ranges ($\pm 2\sigma$) are presented in Table 1.

TABLE I
Expected Values for the hCG ELISA Test System
(In mIU/ml - 3rd IS 75/537)

Number	25
Mean	2.9
Standard Deviation	1.4
Expected Ranges ($\pm 2\sigma$)	0.1 - 5.7

Expected levels for hCG during normal pregnancy (3) are listed in Table 2.

TABLE 2
Expected Values for hCG levels (3rd IS 75/537)
during normal pregnancy (in mIU/ml)

1st week	10 - 30
2nd week	30 - 100
3rd week	100 - 1000
4th week	1,000 -10,000
2nd & 3rd month	30,000 - 100,000
2nd trimester	10,000 - 30,000
3rd trimester	5,000 - 15,000

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.

PERFORMANCE CHARACTERISTICS

A. Precision

The within and between assay precisions of the hCG AccuBind™ ELISA were determined by analyses on three different levels of control sera. The number (N), mean value (X), standard deviation (σ) and coefficient of variation (C.V.) for each of these control sera are presented in Table 3 and Table 4.

TABLE 3
Within Assay Precision (Values in mIU/ml)

Sample	N	X	σ	C.V.
Level 1	20	2.8	0.15	5.4%
Level 2	20	15.2	0.65	4.2%
Level 3	20	178.0	10.50	5.9%

TABLE 4
Between Assay Precision* (Values in mIU/ml)

Sample	N	X	σ	C.V.
Level 1	10	3.1	0.17	5.5%
Level 2	10	15.4	0.81	5.3%
Level 3	10	185.6	11.10	6.0%

*As measured in ten experiments in duplicate.

B. Accuracy

This hCG AccuBind™ ELISA test system was compared with a reference radioimmunoassay. Biological specimens from normal and pregnant populations were assayed. The total number of such specimens was 110. The least square regression equation and the correlation coefficient were computed for the hCG ELISA in comparison with the reference method. The data obtained is displayed below..

Method	Mean (x)	Least Square Regression Analysis	Correlation Coefficient
This Method	14.8	y = 0.081 + 0.93(x)	0.989
Reference	15.1		

Only slight amounts of bias between the hCG ELISA 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.

C. Sensitivity

The hCG AccuBind™ ELISA test system has a sensitivity of 0.02 mIU. This is equivalent to a sample containing 0.8 mIU/ml hCG concentration.

D. Specificity

The cross-reactivity of the hCG AccuBind™ ELISA 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 chorionic gonadotropin needed to produce the same absorbance.

Substance	Cross Reactivity	Concentration
Chorionic Gonadotropin (hCG)	1.0000	----
β-hCG subunit	< 0.0001	1000ng/ml
Follitropin (FSH)	< 0.0001	1000ng/ml
Lutropin Hormone (LH)	< 0.0001	1000ng/ml
Thyrotropin (TSH)	< 0.0001	1000ng/ml

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Revision: B **Date:102506**

Cat #: *825-300*

Size		96(A)	192(B)
Reagent (fill)	A)	1ml set	1ml set
	B)	1 (13ml)	2 (13ml)
	C)	1 plate	2 plates
	D)	1 (20ml)	1 (20ml)
	E)	1 (7ml)	2 (7ml)
	F)	1 (7ml)	2 (7ml)
	G)	1 (8ml)	2 (8ml)

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