Monobind Inc.
Lake Forest, CA 92630, USA

AccuBind
ELISA Microwells

Free ß-Subunit Human Chorionic Gonadotropin (fß-hCG) Test System

Product Code: 2025-300

1.0 INTRODUCTION

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

2.0 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 potential diagnosis *of early* pregnancy disorders. Free ß-hCG subunit testing has improved the diagnostic probability of abnormal pregnancy/disease states (1).

Patients with trophoblastic diseases produce ordinary and irregular forms of hCG; e.g. nicked hCG, hCG missing the *ß-subunit* C-terminal segment, hyperglycosylated hCG and free *ß* subunit. On the other hand common epithelial tumors of the urogenital tract frequently express the free *ß*-Subunit of hCG with no concomitant expression of its heterodimer partner, the common α-subunit of the glycoprotein hormone. While most hCG assays do a very good job of monitoring the normal pregnancies, still there needs to be a system of differential diagnosis of ovarian tumors, epithelial tumors and trophoblastic malfunctions. That is where determination of free α-subunit, free β-subunit, nicked hCG and nonnicked hCG etc are of individual value.

Although fß-hCG normally constitutes less than 1% of the total hCG concentrations in normal pregnancy, it constitutes a significant part (as much as 26% of hCG) in trophoblast disease (2,3). There is also increasing evidence that free beta subunit may be better than total hCG measurement in assessing Down's Syndrome (4).

In this method, fß-hCG calibrator, patient specimen or control is first added to a streptavidin coated well. Biotinylated monoclonal antibody (specific for fß-hCG) is added and the reactants mixed. Reaction between the fß-hCG antibody and native fß-hCG forms complex that binds with the streptavidin coated to the well. The excess serum proteins are washed away via a wash step. Another enzyme labeled monoclonal antibody specific to fβ-hCG is added to the wells. The enzyme labeled antibody binds to the fβ-hCG already immobilized on the well through its binding with the biotinylated monoclonal antibody. Excess enzyme is washed off via a wash step. A color is generated by the addition of a substrate. The intensity of the color generation is directly proportional to the concentration of the fβ-hCG in the sample.

The employment of several serum references of known free β-Chorionic Gonadotropin levels permits the 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 free β- Chorionic Gonadotropin concentration.

3.0 PRINCIPLE

Immunoenzymometric sequential assay (TYPE 4):
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-fß-hCG antibody. Upon mixing monoclonal biotinylated antibody, and a serum containing the native antigen, reaction results between the native antigen and the antibody, forming an antibody-antigen complex. Simultaneously the biotin attached to the antibody binds to the streptavidin coated on the microwells resulting in a complete immobilization of the complex. This interaction is illustrated below:

$$AgB_{(fβhCG)B} + P^{BtnP}AbB_{(m)B} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} AgB_{(fβhCG)B}\text{-}P^{BtnP}AbB_{(m)B}$$

$^{PBtnP}AbB_{(m)B}$ = Biotinylated Monoclonal Antibody (Excess Quantity)

$AgB_{I(fβhCG)B}$ = Native Antigen (Variable Quantity)

$AgB_{(fβhCG)B}\text{-}P^{BtnP}AbB_{(m)B}$= Antigen-Antibody complex (Variable Quant.)

k_a = Rate Constant of Association

k_{-a} = Rate Constant of Disassociation

$AgB_{(fβhCG)B}\text{-}P^{BtnP}AbB_{(m)\,B}+B_{\,BU}\underline{Streptavidin}UB_{C.W.\,B} \Rightarrow$ I<u>mmobilized complex</u> (IC)

U<u>Streptavidin</u>$UB_{C.W.B}$ = Streptavidin immobilized on well

I<u>mmobilized complex (IC)</u>U = Ag-Ab bound to the well

After a suitable incubation period, the antibody-antigen bound fraction is separated from unbound antigen by decantation or aspiration. Another antibody (directed at a different epitope) labeled with an enzyme is added. Another interaction occurs to form an enzyme labeled antibody-antigen-biotinylated-antibody complex on the surface of the wells.

$$(IC) + {}^{Enz}AbB_{(x\text{-}fβhCG)B} \underset{k_{-b}}{\overset{k_b}{\rightleftharpoons}} {}^{Enz}AbB_{(x\text{-}fβhCG)B}\text{ - IC}$$

$^{BEnz}{}_{Ab(x\text{-}fb\text{-}hCG)B}$ = Enzyme labeled Antibody (Excess Quantity)

$^{Enz}AbB_{(x\text{-}fβhCG)B}$ – IC = Antigen-Antibodies Complex

k_b = Rate Constant of Association

k_{-b} = Rate Constant of Dissociation

Excess enzyme is washed off via a wash step. A suitable substrate is added to produce color measurable with the use of a microplate spectrophotometer. The enzyme activity on the well is directly proportional to the native free 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. fß-hCG Calibrators – 1ml/vial - Icons A-F
Six (6) vials of references fß-hCG Antigen at levels of 0(**A**), 2(**B**), 5(**C**), 10(**D**), 25(**E**) and 50(**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 1P^{stP} IRP (75/551).
Conversion to mass units = One (1) mIU/ml is equivalent to 1 ng/ml

B. fβ-hCG Biotin Reagent – 13ml/vial - Icon
One (1) vial containing biotin labeled monoclonal mouse IgG in buffer, dye, and preservative. Store at 2-8°C.

C. fβ-hCG Enzyme Reagent – 13ml/vial - Icon
One (1) vial containing Enzyme (HRP) labeled monoclonal mouse IgG in buffer, dye, and preservative. Store at 2-8°C.

D. 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.

E. Wash Solution Concentrate – 20 ml - Icon
One (1) vial containing 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) bottle containing tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.

G. Substrate B – 7ml/vial - Icon S^B
One (1) bottle containing hydrogen peroxide ($HB_{2B}OB_{2B}$) in buffer. Store at 2-8°C.

H. Stop Solution – 8m/vial - Icon STOP
One (1) bottle containing 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: Opened reagents are stable for sixty (60) days when stored at 2-8°C. **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: See end of this product insert for various configurations of reagents by kit size.

4.1 Required But Not Provided:
1. Pipette capable of delivering 25µl and 50µl volumes 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. 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.

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 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.

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 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 for samples. Centrifuge the specimen to separate the serum from the cells.

Samples may be refrigerated at 2-8^{0}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^{0}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, medium and high ranges of the dose response curve 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.

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 at 2-30°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.

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 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 the fβhCG Biotin Reagent to each well. **It is very important to dispense all reagents close to the bottom of the coated well.**
4. Swirl the microplate gently for 20-30 seconds to mix and cover.
5. Incubate 30 minutes at room temperature.
1. Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.
7. 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.**
8. Add 0.100 ml (100µl) of the fßhCG Enzyme Reagent to each well.
   DO NOT SHAKE THE PLATE AFTER ENZYME ADDITION
9. Cover and incubate 30 minutes at room temperature.
10. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.
11. 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.**

12. 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 PLATE AFTER SUBSTRATE ADDITION
13. Incubate at room temperature for fifteen (15) minutes.
14. Add 0.050ml (50µl) of stop solution to each well and mix gently for 15-20 seconds. **Always add reagents in the same order to minimize reaction time differences between wells.**
15. 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.**

10.0 CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of fß-Chorionic Gonadotropin 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 fß-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 fß-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 (0.438) intersects the dose response curve at (6.0 mIU/ml) fß-hCG concentration (See Figure 1).

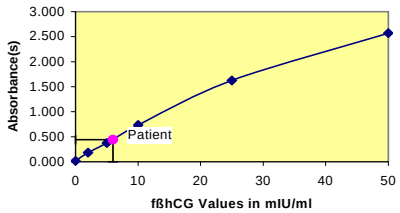
Note: Computer data reduction software designed for ELISA assays may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

EXAMPLE 1

Sample I.D.	Well Position	Absorbance	Mean Absorbance (B)	Concentration
Cal A	A1	0.012	0.015	0
	B1	0.017		
Cal B	C1	0.181	0.181	2
	D1	0.181		
Cal C	E1	0.377	0.374	5
	F1	0.371		
Cal D	G1	0.737	0.734	10
	H1	0.731		
Cal E	A2	1.639	1.624	25
	B2	1.608		
Cal F	C2	2.642	2.571	50
	D2	2.501		
Control	E2	0.113	0.108	***1.1***
	F2	0.102		
Patient	G2	0.447	0.438	***6.0***
	H2	0.430		

*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.

Figure 1



11.0 Q.C. PARAMETERS

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

1. Maximum Absorbance (Calibrator 'F') = >1.3
2. Maximum Absorbance (Calibrator 'A') = U≤U 0.1
3. 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 fß-hCG concentrations above 50 mIU/ml may be diluted (for example 1/10) with normal male serum (fß-hCG < 1 mIU/ml) 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

Serum free β-hCG (as intact hCG) increases rapidly in normal pregnancy reaching maximum levels of approximately 60ng/ml at eight-nine weeks of gestation. This is followed by a gradual decline during the next eleven to twelve weeks. The ration of free β-hCG to intact hCG reaches 1% at five weeks of pregnancy and remains constant at approximately 0.5% (w/w) for the remaining twenty-two weeks (2).

The use of free β-hCG in combination with AFP levels as a screening protocol for Down syndrome (Trisomy 21) has been promoted to achieve high detection efficiency with low false positive rates (5).

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 precisions of the fß-hCG AccuBind™ ELISA test system were determined by analyses on three different levels of control sera. The number, mean value, standard deviation and coefficient of variation for each of these control sera are presented in Table 3 and Table 4.

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

Sample	N	X	σ	C.V.
Level 1	20	3.5	0.15	4.3%
Level 2	20	10.5	0.55	5.2%
Level 3	20	49.6	1.50	3.0%

TABLE 3
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	11.2	0.71	6.3%
Level 3	10	48.5	1.75	3.6%

*As measured in ten experiments in duplicate.

14.2 Sensitivity

The fß-hCG AccuBind™ ELISA test method has a sensitivity of 0.0007 mIU. This is equivalent to a sample containing 0.026mIU/ml fß-hCG concentration.

14.3 Specificity

The cross-reactivity of the fß-hCG AccuBind™ ELISA test method 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
fß-hCG subunit	1.0000
Intact Chorionic Gonadotropin (hCG)	< 0.0001
Follicle Stimulating Hormone (FSH)	< 0.0001
Luteinizing Hormone (LH)	< 0.0001
Thyroid Stimulating Hormone (TSH)	< 0.0001

15.0 REFERENCES

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Size		96(A)	192(B)
Reagent (fill)	A)	1ml set	1ml set
	B)	1 (13ml)	2 (13ml)
	C)	1 (13ml)	2 (13ml)
	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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