

Instruction Manual No. M-410

**Free Beta Human Chorionic Gonadotropin
(Free β HCG)**

**For Quantitative Determination of
Free-Beta HCG In Human Serum**

For In Vitro Research Use Only

**Free Beta Human Chorionic Gonadotropin (Free-Beta HCG)
ELISA KIT Cat. No. 0410 (96 Tests)**

Kit Components	96 Tests
Anti-Beta HCG Coated Strip plates (96 wells). Cat.# 411	1 Plate
Std. A , Cat. # 412, 0 mIU/ml (sample diluent), 11 ml	1 vial
Std. B , Cat. # 413, 2.5 mIU/ml, 0.3 ml	1 vial
Std. C , Cat. # 414, 5 mIU/ml, 0.3 ml	1 vial
Std. D , Cat. # 415, 10 mIU/ml, 0.3 ml	1 vial
Std. E , Cat. # 416, 30 mIU/ml, 0.3 ml	1 vial
Std. F , Cat. # 417, 100 mIU/ml, 0.3 ml	1 vial
Standards are provided in BSA-containing diluent and calibrated against WHO IRP 75/551 (1 mIU/ml=1 ng/mL).	
Free beta Low Control Serum (see conc on the vial)	4 1 0 C 1
Free beta High Control Serum (see conc on the vial)	4 1 0 C 2
Wash Buffer (20X), 50 ml	W B - 2 0
Anti-free-beta HCG-HRP Conjugate , Cat # 4 1 8 , 11 ml ready-to-use	1 bottle
HRP Substrate Solution A , 11 ml	Cat. # 410SA
HRP Substrate Solution B , 11 ml	Cat. # 410SB
Stop solution, Cat. # T - 1 0 , 10 ml	1 bottle
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Introduction

Free beta HCG is a hormone produced by the placenta during pregnancy. The hormone is present in blood and urine around seven to 13 days following implantation of the fertilized ovum. Structurally intact HCG molecule consists of two non-covalently linked polypeptide subunits, the alpha and beta chain subunits. Measurements of intact HCG and of the alpha subunit of HCG appear to give similar results in blood and urine but not the levels of beta subunit.

In normal second trimester maternal sera, the level of intact HCG ranges from 20,000-50,000 mIU/ml. In contrast, the level of either free alpha or free beta-HCG is on average one half of 1% of HCG levels. HCG and the free subunits appear not to be useful as serological markers for nontrophoblastic tumors; however, the absolute increase of Beta-HCG level in choriocarcinoma patients clearly differentiates it from normal pregnancy.

Recent studies showed a significant increase in the level of free HCG-beta in trisomy 21 cases as compared with controls. Hence, it has been suggested that free Beta-HCG subunit assay in combination of maternal serum AFP could be effective in a screening for trisomy 21.

PERFORMANCE CHARACTERISTICS

1. DETECTION LIMIT

Based on sixteen replicates determinations of the zero standard, the minimum concentration of human free β -HCG detected using this assay is 0.5 mIU/ml. The detection limit is defined as the value deviating by $2\pm$ SD from the zero standard.

2. PRECISION

Intra-assay precision:

Three serum samples (free β -HCG concentrations 16.5, 34, 90.1 mIU/ml) were run in 10 replicates. The samples showed good intra-assay precision with % CV of 10.3, 11.5, and 8.8, respectively.

Inter-assay precision:

Three serum samples were run in duplicate in 10 independent assays. The samples showed good inter-assay precision (9.2-10.3% CV). The actual values were: mean 17.4, 35.8, and 96.6 mIU/ml).

3. SPECIFICITY

The specificity of free β -HCG ELISA kit was determined by measuring interference from high concentrations of the following:

Hormone tested	Conc. (mIU/ml)	Color intensity produced Equivalent of free β-HCG (mIU/ml)
HCG	10,000	2
	1000	<2
	100	0
Alpha-HCG	100	0
hLH	200	<2
	80	0
TSH	75	0
FSH	200	0

References:

1. Birken S. Canfield RE Chemistry & Immunochemistry of HCG. In; Segal SJ Ed. Chorionic Gonadotropin. NY ; plenum Press: 65-88, 1980.
2. Cole LA. Immunoassay of HCG it's free Subunit and Metabolites. Clinical Chem. 43:12 2233-2243, 1997.
3. Ozturk M et al, Physiological Studies of HCG, alpha hCG, b-hCG as measured by Specific Monoclonal Immunoradiometric assays. Endocrinology. 120: 549-558, 1987
4. MacriJN et al Maternal Serum Down Syndrome Screening; Free protein is a more effective marker than HCG. Am. J. Obstet. Gynecol. 163: 1248-1253, 1990.

STORAGE AND STABILITY

The microtiter well plate and all other reagents are stable at 2-8°C until the expiration date printed on the label. The whole kit stability is usually 6 months from the date of shipping under appropriate storage conditions.

TEST PROCEDURE (ALLOW ALL REAGENTS TO REACH ROOM TEMPERATURE BEFORE USE). Prepare 1x wash buffer by diluting 1:20 with water (**dilute 50 ml in 950 ml water**). Store at 4°C until use.

1. Label or mark the microtiter well strips to be used on the plate. Dispense 200-300 μ l of 1x wash buffer or water to all wells. Mix for **5 seconds** and discard or aspirate the solution. The step should be done just before adding the samples, do not allow the wells to dry at any time during the assay.
2. Pipet **25 μ l** of standards, control, and serum samples into appropriate wells in *duplicate*.
3. Dispense **100 μ l** enzyme conjugate into each well. Mix gently for 5-10 seconds. Cover the plate and incubate at room temp. for **60 minutes**.
4. Aspirate and wash the wells **5 times** with wash buffer. Failure to wash the wells properly will lead to high blank or zero values. If washing manually, plate must be tapped over paper towel between washings to ensure proper washing.
5. Aspirate and wash the wells **5 times** with wash buffer, as above.
6. Premix HRP substrate **Solution A and B** (1:1 v/v) and dispense 200 μ l per well (prepare 1.8 ml per strip or need 20 ml for full plate). Do not keep mixed solution for more than 10-20 min and prepare only in required amounts. Make sure that TMB solution is at room temp before mixing and dispensing into the plate. Mix the plate gently for 5-10 seconds. Cover the plate and incubate at room temp. for **30 minutes**. Blue color develops in standards and positive wells.
7. Stop the reaction by adding **50 μ l of stopping** solution to all wells at the same timed intervals as in step 6 (blue color turns yellow). Mix gently. Measure absorbance at 450 nm within 15-30 min.

NOTES

Read instructions carefully before the assay. Do not allow reagents to dry on the wells. Careful aspiration of the washing solution is essential for good assay precision.

Since timing of the incubation steps is important to the performance of the assay, pipet the samples without interruption and it should not exceed 5 minutes to avoid assay drift. If more than one plate is being used in one run, it is recommended to include a standard curve on each plate. The unused strips should be stored in a sealed bag at 4°C.

Addition of the HRP substrate solution starts a kinetic reaction, which is terminated by dispensing the stopping solution. Therefore, keep the incubation time for each well the same by adding the reagents in identical sequence. Plate readers measure absorbance vertically. Do not touch the bottom of the wells.

DILUTION OF SAMPLES

Serum samples containing more than 100 mIU/ml free beta-HCG must be diluted with the zero standard (standard A) and the results obtained should be multiplied by the appropriate dilution factor.

CALCULATION OF RESULTS

Calculate the mean absorbance for each duplicate. Draw the standard curve on log-log graph paper by plotting net absorbance values of standards against appropriate free beta HCG concentrations. Read off the free beta HCG concentrations of the control and patient samples.

Expected Values for free β -HCG

1. In early pregnancy, free β -HCG conc. was 10-80 mIU/ml. The free β -HCG/intact HCG ratio was found to be 3.08-28 %. After 6-7 weeks, free β -HCG and the ratio value declined. During the 2nd and 3rd trimester, a constant ratio was observed about 1%.
2. Serum samples from 40 normal subjects were 0.4 mIU/ml (99.5% values).
3. Serum intact HCG and free β -HCG levels in sera from patients with gestational choriocarcinoma were reported (Ozuturk et al Endocrinol. 120, 499-508).

Patient #	HCG (mIU/ml)	α -HCG (mIU/ml)	β -HCG (mIU/ml)
1	210,000	112	8000
2	22,195	20	1300
3	6,840	1	232
4	36,000	44	3900
5	4,200	2	350

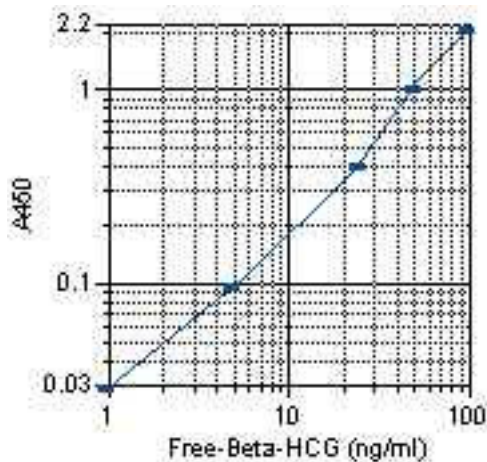
4. In the study of 479 maternal sera, 29 cases of trisomy 21 and 450 controls were test. A significant increase in the level of free- β -hCG in Trisomy 21 cases were observed⁴:

Weeks Gestation	Control (mIU/ml)	Trisomy 21(mIU/ml)
14 - 16	16.3	34.7
17 - 18	10.9	19.9

WORKSHEET OF TYPICAL ASSAY

Wells	Stds/samples	Mean A_{450nm}	Calculated Conc. (mIU/ml)
A1, A2	Std. A (0 mIU/ml)	0.007	
B1, B2	Std. B (2.5 mIU/ml)	0.067	
C1, C2	Std. C (5 mIU/ml)	0.162	
D1, D2	Std. D (10 mIU/ml)	0.363	
E1, E2	Std. E (30 mIU/ml)	1.105	
F1, F2	Std. F (100 mIU/ml)	2.648	
G1, G2	Sample 1	0.744	19.8

NOTE: These data are for demonstration purpose only. A complete standard curve must be run in every assay to determine sample values. Each laboratory should determine their own normal reference values.



A typical std. assay curve (do not use this for calculating sample values)

PRINCIPLE OF THE TEST

Free β -HCG ELISA kit is based on sequential binding of Free β -HCG from patient samples to two antibodies, one immobilized on microtiter well plates, and other conjugated to the enzyme horseradish peroxidase. After a washing step, chromogenic substrate is added and color developed. The enzymatic reaction (color) is directly proportional to the amount of Free β -HCG present in the sample. Adding stopping solution terminates the reaction. Absorbance is then measured on a microtiter well ELISA reader at **450 nm**. The unknown sample values are then read-off the standard curve.

MATERIALS AND EQUIPMENT REQUIRED

Adjustable micropipet (20-100 μ l) and Multichannel pipet with disposable plastic tips. Reagent troughs. Plate washer (recommended) and ELISA plate Reader.

PRECAUTIONS

The HCG ELISA kit is intended for *in vitro* research use only. The reagents contain prolcin-300 as preservative; necessary care should be taken when disposing solutions. Some components have been tested using FDA-approved methods and has been found negative for antibody to human immuno-deficiency virus (HIV-I, HIV-II), antibody to Hepatitis C, and Hepatitis B surface antigen (HBsAg). No known test method can offer total assurance that HIV-I, HIV-II, hepatitis B and C virus or other infectious agents are absent. Handle these reagents as if they were potentially infectious. Information on handling human serum is provided in the CDC/NIH manual "Biosafety in Microbiological and Biomedical Laboratories" (U.S.A. HHS publication No.(NIH 88-8395.).

Applicable **MSDS**, if not already on file, for the following reagents can be obtained from ADI or the web site.

TMB (substrate), H₂SO₄ (stop solution), and Prolcin-300 (0.1% v/v in standards, sample diluent and HRP-conjugates).

SPECIMEN COLLECTION AND HANDLING

Collect blood by venipuncture, allow to clot, and separate the serum by centrifugation at room temperature. Do not heat inactivate the serum.. If sera can not be immediately assayed, these could be stored at -20°C for up to six months. Avoid repeated freezing and thawing of samples. No preservatives should be added to the serum. Sodium azide, an inhibitor of peroxidase, must not be present in the samples.