Free Beta-Subunit of Human Chorionic Gonadotropin ELISA Kit Protocol

INTENDED USE

For the quantitative determination of free beta subunit of human chorionic gonadotropin (free Beta-hCG) concentration in human serum. **FOR RESEARCH ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES**

LIMITATIONS OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the package insert instructions and will adherence to good laboratory practice.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
3. Serum samples demonstrating gross lipemia, gross hemolysis, or turbidity should not be used with this test.

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INTRODUCTION AND PROTOCOL OVERVIEW

Human Chorionic Gonadotropin (hCG) is a glycoprotein hormone normally produced by placenta during pregnancy. The hormone is present in blood and urine around seven to thirteen days following implantation of the fertilized ovum. Structurally intact hCG molecules consist of two non-covalently linked polypeptide subunits, the alpha and beta chain subunits. Measurement of intact hCG and of the alpha subunit of hCG appears to give similar results in blood and urine but not the levels of beta subunit. In the normal second-trimester maternal sera, the level of intact hCG range from 20,000 mIU/ml to 50,000 mIU/ml (1 ng = 15 mIU). In contrast, the levels of either free alpha- or free Beta-hCG are 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.

PRINCIPLE OF THE TEST

The free Beta-hCG ELISA test is based on the principle of a solid phase enzyme-linked immunosorbent assay.1 The assay system utilizes a unique monoclonal antibody directed against a distinct antigenic determinant on the free Beta-hCG. Mouse monoclonal anti-free-Beta-hCG antibody is used for solid phase immobilization (on the microtiter wells). A goat anti whole hCG antibody is in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample is allowed to react sequentially with the two antibodies, resulting in the free Beta-hCG molecules being sandwiched between the solid phase and enzyme-linked antibodies. After two separate 30 minute incubations at 37 °C, the wells are washed with water to remove unbound labeled antibodies. A solution of TMB Reagent is added and incubated for 20 minutes, resulting in the development of a blue color. The color development is stopped with the addition of Stop Solution changing the color to yellow. The concentration of Beta-hCG is directly proportional to the color intensity of the test sample. Absorbance is measured spectrophotometrically at 450 nm.

STORAGE

Unopened test kits should be stored at 2-8 ºC upon receipt and the microtiter plate should be kept in a sealed bag with desiccants to minimize exposure to damp air. Opened test kits will remain stable until the expiration date shown, provided it is stored as described above. A microtiter plate reader with a bandwidth of 10 nm or less and an optical density range of 0-2 OD or greater at 450 nm wavelength is acceptable for use in absorbance measurement

LIST OF COMPONENTS

Materials Provided with the Kit:

- Murine Monoclonal Anti-free-beta-hCG-coated microtiter wells.
- Set of Reference Standards: 0, 2.5, 5, 10, 25, and 50 ng/ml, lyophilized.
- Zero Buffer (Sample diluent), 13 ml.
- Enzyme Conjugate Reagent, 18 ml.
- TMB Reagent (One-Step), 11 ml.
- Stop Solution (1N HCl), 11 ml.

Materials required but not provided:

- Precision pipettes: 50 µl, 100 µl, 150 µl, and 1.0 ml.
- Disposable pipette tips.
- Distilled water.
- Vortex mixer or equivalent.
- Absorbent paper or paper towel.
- Graph paper.
- Microtiter plate reader.

SPECIMEN COLLECTION AND PREPARATION

Serum should be prepared from a whole blood specimen obtained by acceptable medical techniques. This kit is for use with serum samples without additives only.

REAGENT PREPARATION

1. All reagents should be brought to room temperature (18-25 ºC) before use.
2. Reconstitute each lyophilized standard with 1.0 ml distilled water. Allow the reconstituted material to stand for at least 20 minutes and mix gently. Reconstituted standards will be stable for up to 30 days when stored sealed at 2-8 ºC.

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 50 µl of standards, specimens, and controls into appropriate wells.
3. Dispense 100 µl of Zero Buffer into each well.
4. Thoroughly mix for 30 seconds. It is very important to mix them completely.
5. Incubate at 37 ºC for 30 minutes.
6. Remove the incubation mixture by flicking plate contents into a sink.
7. Rinse and flick the microtiter wells 5 times with distilled or deionized water. (Please do not use tap water.)
8. Strike the wells sharply onto absorbent paper or paper towels to remove all residual water droplets.
9. Dispense 150 µl of Enzyme Conjugate Reagent into each well. Gently mix for 10 seconds.
10. Incubate at 37 ºC for 30 minutes.
11. Remove the incubation mixture by flicking plate contents into a waste container.
12. Rinse and flick the microtiter wells 5 times with distilled or deionized water. (Please do not use tap water.)
13. Strike the wells sharply onto absorbent paper or paper towels to remove all residual water droplets.
14. Dispense 100 µl of TMB Reagent into each well. Gently mix for 10 seconds.
15. Incubate at room temperature for 20 minutes.

16. Stop the reaction by adding 100 µl of Stop Solution to each well.
17. Gently mix for 30 seconds. It is important to make sure that all the blue color changes to yellow color completely.
18. Read optical density at 450 nm with a microtiter well reader within 15 minutes.

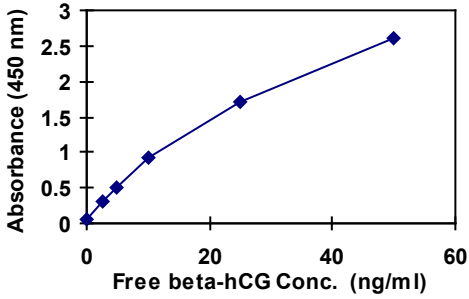
CALCULATION OF RESULTS

1. Calculate the mean absorbance value (A450) for each set of reference standards, controls and patient samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on graph paper, with absorbance values on the vertical or Y axis, and concentrations on the horizontal or X axis.
3. Use the mean absorbance values for each specimen to determine the corresponding concentration of free Beta-hCG in ng/ml from the standard curve.

EXAMPLE OF STANDARD CURVE

Results of a typical standard run with optical density readings at 450 nm shown in the Y axis against free Beta-hCG concentrations shown in the X axis. This standard curve is for the purpose of illustration only, and should not be used to calculate unknowns. Each user should obtain his or her own data and standard curve..

ß-hCG (ng/ml)	Absorbance (450 nm)
0	0.061
2.5	0.296
5.0	0.498
10.0	0.929
25.0	1.711
50.0	2.613



STANDARDIZATION

For intact hCG, 1 ng is approximately equivalent to 15 mIU (WHO, 1st IRP 75/537). For free β-hCG subunit, since there is no WHO standardization, we tested the free β-hCG against hCG ELISA kit, and found 1 ng of free β-hCG equals to 0.1 mIU in terms of hCG immunological activity.

EXPECTED VALUES

The following information is cited from references #4, 5, 6, and 7:

1. hCG and Free β-hCG Subunit Levels in Normal Pregnancy

A logarithmic increase in the serum concentration of hCG was observed from 5-8 weeks of gestation (2,600 ng/ml to 33,000 ng/ml) as defined by last menstrual period; thereafter, hCG values decreased. Similarly, free β-hCG levels increased rapidly to reach maximum levels (~60 ng/ml) at 8-9 weeks of pregnancy, followed by a gradual decline during the next 11-12 weeks of gestation.
At 5 weeks of gestation, the ratio of free β-hCG to intact hCG is approximately 1.0 % (w/w). Thereafter, this ratio remains remarkably constant over 22 weeks of gestation (~ 0.5 % w/w).

2. hCG and Free Subunits Levels in Gestational Choriocarcinoma

Free α and free β-subunits and hCG levels were measured in five patients with untreated gestational choriocarcinoma. The concentrations in serum are shown in the following table:

Patient Number	hCG(ng/ml)	α-hCG(ng/ml)	β-hCG(ng/ml)
1	210,000	112	8,000
2	22,195	20	1,300
3	6,840	1	232
4	36,000	44	3,900
5	4,200	2	350

The levels of free α-hCG were low, ranging from 1-112 ng/ml, whereas hCG levels ranged from 4,200 to 210,000 ng/ml (1 ng ≈ 15 mIU). In contrast, free β-hCG concentrations were found to be markedly elevated in choriocarcinoma.

SENSITIVITY

The minimum detectable concentration of this Beta-hCG in this assay is estimated to be 0.25 ng/ml.

REFERENCES

3. Engall, E., Enzyme immunoassay ELISA and EMIT. In: Van Vunakis, H. and Langone, J.J. (eds.), Methods in Enzymology, Academic Press, New York, 1980; 70: 419-439.
4. Densem, J., and Wald, N.J., The stability of blood samples for the measurement of the free β subunit of chorionic gonadotrophin. Prenat. Diagn., 1995; 15: 94-95.
5. Hay, D.L., Placental histology and the production of human choriogonadotrophin and its subunits in pregnancy. Br. J Obstet. Gynaecol., 1988; 95: 1268-1275.
6. Ozturk, M., Bellet, D., Manil, L., et. al., Physiological studies of human chorionic gonadotropin (hCG), αhCG, and βhCG as measured by specific monoclonal immunoradiometric assays. Endocrinology, 1987; 120: 549-558.
7. Ozturk, M., Berkowitz, R., Goldstein, D., Bellet, D., Wands, J. R., Differential production of human chorionic gonadotropin and free subunits in gestational trophoblastic disease. Am. J. Obstet. Gynecol.,1988; 158:193-198.
8. Cole, L.A., Hartle, R.J., Laferla, J.J., et al., Detection of the free beta subunit of human chorionic gonadotropin (hCG) in cultures of normal and malignant trophoblast cells, pregnancy sera, and sera of patients with choriocarcinoma. Endocrinology, 1983; 113:1176-1178.
9. Gaspard, U.J., Reuter, A.M., Devìlle, J-L, et al., Serum concentration of human chorionic gonadotropin and its alpha and beta subunits. Clin. Endocrinol. (OXF), 1980; 13: 319-329.

ASSAY DIAGRAM