Instructions for Use

Free beta-hCG ELISA

Enzyme immunoassays for the quantitative determination of free beta-hCG chain in human serum.

REF **RE52031**

Σ **96**

2-8 °C

EU: IVD CE

U.S.: *For research use only.*
Not for use in diagnostic procedures.

I B L I N T E R N A T I O N A L G M B H
Flughafenstrasse 52a
D-22335 Hamburg, Germany
Phone: +49 (0)40-53 28 91-0
Fax: +49 (0)40-53 28 91-11
IBL@IBL-International.com
www.IBL-International.com

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For In Vitro Diagnostic Use Only

Store at 2 to 8°C.

1 INTENDED USE

For the quantitative determination of free beta subunit of human chorionic gonadotropin (free β-hCG) concentration in human serum.

2 INTRODUCTION

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 α- or free β-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 β-hCG level in choriocarcinoma patients clearly differentiates it from normal pregnancy.

3 PRINCIPLE OF THE TEST

The free β-hCG ELISA test is based on the principle of a solid phase enzyme-linked immunosorbent assay.[i] The assay system utilizes a unique monoclonal antibody directed against a distinct antigenic determinant on the free β-hCG. Mouse monoclonal anti-free-β-hCG antibody is used for solid phase immobilization (on the microtiter wells). A goat 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 β-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 β-hCG is directly proportional to the color intensity of the test sample. Absorbance is measured spectrophotometrically at 450 nm.

4 REAGENTS

4.1 Materials provided with the kit:

1. **Antibody-Coated Wells** (1 plate, 96 wells)
   Microtiter Wells coated with monoclonal anti-free-β-hCG.
2. **Reference Standard Set** (1.0 ml/vial)
   Contains 0, 2.5, 5, 10, 25, and 50 ng/ml of β-hCG in bovine serum with preservatives, lyophilized.
3. **Zero Buffer** (13 ml)
   Contains tris buffer with preservatives.
4. **Enzyme Conjugate** Reagent (18 ml)
   Contains goat anti-whole hCG conjugated to horseradish peroxidase with preservatives.
5. **TMB Reagent** (11 ml)
   Contains 3, 3', 5, 5' tetramethylbenzidine (TMB) stabilized in buffer solution.
6. **Stop Solution** -1N HCl (11 ml)
   Diluted hydrochloric acid.

4.2 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.

5 WARNINGS AND PRECAUTIONS

1. CAUTION: This kit contains human material. The source material used for manufacture of this component tested negative for HBsAg, HIV 1/2 and HCV by FDA-approved methods. However, no method can completely assure absence of these agents. Therefore, all human blood products, including serum samples, should be considered potentially infectious. Handling should be as defined by an appropriate national biohazard safety guideline or regulation, where it exists.[2-4]
2. Avoid contact with 1N HCl. It may cause skin irritation and burns. If contact occurs, wash with copious amounts of water and seek medical attention if irritation persists.
3. Do not use reagents after expiration date and do not mix or use components from kits with different lot numbers.
4. Replace caps on reagents immediately. Do not switch caps.
5. Do not pipette reagents by mouth.
6. For in vitro diagnostic use.

6 STORAGE CONDITIONS

1. Store the unopened kit at 2-8°C upon receipt and when it is not in use, until the expiration shown on the kit label. Refer to the package label for the expiration date.
2. Keep microtiter plate in a sealed bag with desiccant to minimize exposure to damp air.

7 INSTRUMENTATION

A microtiter well reader with a bandwidth of 10 nm or less and an optical density range of 0 to 3 OD or greater at 450 nm wavelength is acceptable for absorbance measurement.

8 SPECIMEN COLLECTION AND PREPARATION

1. The use of SERUM samples is required for this test.
2. Specimens should be collected using standard venipuncture techniques. Remove serum from the coagulated or packed cells within 60 minutes after collection.
3. Specimens which cannot be assayed within 24 hours of collection should be frozen at –20°C or lower, and will be stable for up to six months.
4. Avoid grossly hemolytic (bright red), lipemic (milky), or turbid samples (after centrifugation).
5. Specimens should not be repeatedly frozen and thawed prior to testing. DO NOT store in “frost free” freezers, which may cause occasional thawing. Specimens which have been frozen, and those which are turbid and/or contain particulate matter, must be centrifuged prior to use.

9 PROCEDURAL NOTES

1. Pipetting Recommendations (single and multi-channel): Pipetting of all standards, samples, and controls should be completed within 3 minutes.
2. All standards, samples, and controls should be run in duplicate concurrently so that all conditions of testing are the same.
3. It is recommended that the wells be read within 15 minutes following addition of Stop Solution.

10 REAGENT PREPARATION

1. All reagents should be brought to room temperature (18-25 °C) 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 °C.

11 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 °C 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 °C 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***.

12 QUALITY CONTROL

Good laboratory practice requires that quality control specimens (controls) be run with each calibration curve to verify assay performance. To ensure proper performance, control material should be assayed repeatedly to establish mean values and acceptable ranges.

It is recommended to use BIOREF control samples according to state and federal regulations. Use controls at both normal and pathological levels.

Example:

BIOREF RM 16; Lot: 24030; 2009-06

Expected values:

Level 1	1.54 – 2.87
Level 2	9.1 – 16.9

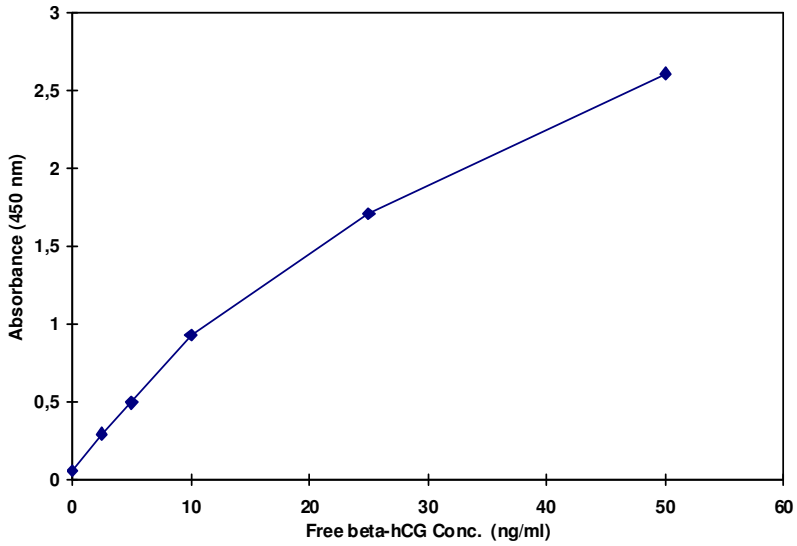
13 CALCULATION OF RESULTS

1. Calculate the mean absorbance value (A_{450}) 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 β-hCG in ng/ml from the standard curve.

14 EXAMPLE OF STANDARD CURVE

Results of a typical standard run with optical density readings at 450 nm shown in the Y axis against free β-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



15 STANDARDIZATION

The concentrations of the Free β HCG Kit standards match the WHO Reference Reagent Human Chorionic Gonadotrophin, Beta Subunit (Purified) (NIBSC code: 99/650)

16 EXPECTED VALUES

The following information is cited from references #9, 11, 13, 14, and 15:

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.

17 PERFORMANCE CHARACTERISTICS

17.1 Sensitivity

The minimal detectable concentration of free β-hCG by this assay is estimated to be 0.25 ng/mL.

17.2 Precision

17.2.1 Intra-Assay Precision

Within-run precision was determined by replicate determinations of three different control sera in one assay. Within-assay variability is shown below:

Serum Sample	1	2	3
Number of Replicates	24	24	24
Mean free β-hCG (ng/mL)	2.8	14	36
Standard Deviation	0.11	0.4	1.5
Coefficient of Variation (%)	3.7	2.6	4.3

17.2.2 Inter-Assay Precision

Between-run precision was determined by replicate measurements of three different control sera in several different assays. Between-assay variability is shown below:

Serum Sample	1	2	3
Number of Replicates	20	20	20
Mean free β-hCG (ng/mL)	3.0	17	37
Standard Deviation	0.10	0.7	0.7
Coefficient of Variation (%)	3.4	4.0	2.0

17.3 Recovery and Linearity Studies

17.3.1 Recovery

Various patient serum samples of known free β-hCG levels were mixed and assayed in duplicate. The average recovery was 96.7%.

	Expected Concentration (ng/ml)	Observed Concentration (ng/ml)	% Recovery
1.	41.22	39.68	96.3
2.	40.61	37.73	92.9
3.	15.07	15.58	103.4
4.	17.44	17.06	97.8
5.	3.389	3.258	96.1
6.	3.352	2.986	89.1
7.	0.493	0.478	97.1
8.	0.436	0.441	101.1
	Average Recovery = 96.7%		

17.3.2 Linearity

Four patient samples were serially diluted with the zero standard in a linearity study. The average recovery was 111.3%.

#	Dilution	Expected Conc. (ng/mL)	Observed Conc. (ng/mL)	% Recovery
1.	Undiluted	----	34.54	----
	1:2	17.27	19.18	111.1
	1:4	8.634	8.822	102.2
	1:8	4.317	4.701	108.9
	1:16	2.159	2.398	111.1
				Mean = 108.3 %
2.	Undiluted	----	47.81	----
	1:2	23.91	23.98	100.3
	1:4	11.95	13.69	114.5
	1:8	5.977	7.538	126.1
	1:16	2.988	3.872	129.6
	1:32	1.494	1.921	128.6
				Mean = 117.6 %
3.	Undiluted	----	42.09	----
	1:2	21.05	22.70	107.9
	1:4	10.52	10.50	99.8
	1:8	5.262	5.519	104.9
	1:16	2.631	2.930	111.4
	1:32	1.315	1.622	123.3
				Mean = 106.0%
4.	Undiluted	----	35.48	----
	1:2	17.74	20.27	114.3
	1:4	8.869	9.424	106.3
	1:8	4.435	5.200	117.3
	1:16	2.217	2.554	115.2
	1:32	1.109	1.367	123.3
				Mean = 113.3%

17.4 Specificity

The following substances were tested for cross-reactivity:

Analytes and Concentration	% Cross Reactivity
Intact hCG (100,000 mIU/ml)	0.5%
Beta-hCG	100.0%
Alpha-hCG (500 ng/ml)	0.0%
LH (500 mIU/ml)	0.0%
TSH (500 µIU/ml)	0.0%
FSH (500 mIU/ml)	0.0%

18 LIMITATIONS OF THE PROCEDURE

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.

The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

Serum samples demonstrating gross lipemia, gross hemolysis, or turbidity should not be used with this test.

The results obtained from the use of this kit should be used only as an adjunct to other diagnostic procedures and information available to the physician.

19 REFERENCES/LITERATURE

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KURZANLEITUNG

REAGENZIEN

1. **Microtiter Wells**: 96 wells, beschichtet mit monoklonalem anti-freies-ß-hCG-Antikörper
2. **Standards** (lyophilisiert): 0, 2.5, 5, 10, 25 und 50 ng/mL
   siehe "Vorbereitung der Reagenzien "
3. **Zero Buffer**, 13 mL, gebrauchsfertig
4. **Conjugate Conjugate** (Enzymkonjugat), 18 mL, gebrauchsfertig,
   anti-hCG-Antikörper mit Meerrettichperoxidase konjugiert
5. **TMB Reagent** (Substratlösung), 11 mL, gebrauchsfertig
6. **Stop Solution** (Stopplösung), 11 mL, gebrauchsfertig

LAGERUNG UND HALTBARKEIT DES KITS

Die ungeöffneten Reagenzien behalten gelagert bei 2 °C bis 8 °C ihre Reaktivität bis zum Verfallsdatum. Nach dem Verfallsdatum die Reagenzien nicht mehr verwenden.
Nach dem Öffnen sollten alle Reagenzien bei 2°C bis 8°C gelagert werden. Die Mikrotiterwells sollten bei 2°C bis 8°C gelagert werden. Der einmal geöffnete Folienbeutel sollte stets sehr sorgfältig wieder verschlossen werden. Unter den beschriebenen Lagerbedingungen behalten geöffnete Kits ihre Reaktivität bis zum angegebenen Verfallsdatum.

VORBEREITUNG DER REAGENZIEN

1. Bevor Sie mit dem Test beginnen, sollten sich alle Proben und Reagenzien auf Raumtemperatur (18-25°C) erwärmt haben.
2. **Standards:**
   Rekonstruieren Sie den lyophilisierten Inhalt der Standardröhrchen mit 1.0 mL Aqua dest, lassen Sie diese 20 Minuten stehen. Die Standards sind bis zu 30 Tagen stabil, wenn sie verschlossen bei 2°C - 8°C gelagert werden.

TESTDURCHFÜHRUNG

1. Setzen Sie die gewünschte Menge an beschichteten Mikrotiterwells in die Mikrotiterplatte.
2. Pipettieren Sie 50 µL Standards, Kontrollen oder Serumproben in die dafür vorgesehenen Wells.
3. Geben Sie 100 µL vom Zero Buffer in jedes Well.
4. Für 30 Sekunden **gründlich** mischen. (Es ist sehr wichtig komplett durchzumischen).
5. Inkubieren Sie für 30 Minuten bei 37°C.
6. Strips 5-mal mit je 300 µL Aqua dest pro Well waschen, abschütten und umgedreht auf saugfähigem Papier fest ausklopfen.
7. Pipettieren Sie 150 µL des Enzymkonjugates in jedes Well. Vorsichtig 5 Sekunden schütteln.
8. Inkubieren Sie 30 Minuten bei 37°C.
9. Streifen 5-mal mit je 300 µL Aqua dest pro Well waschen, abschütten und umgedreht auf saugfähigem Papier fest ausklopfen.
10. Pipettieren Sie je 100 µL TMB Substrate Solution in jedes Well. Die Platte 5 Sekunden vorsichtig mischen.
11. Bei Raumtemperatur 20 Minuten inkubieren.
12. Die Reaktion wird gestoppt, wenn Sie 100 µL der Stopplösung jedem Well hinzufügen.
13. Vorsichtig für 30 Sekunden mischen. **Achtung:** Die blaue Färbung sollte sich komplett in eine gelbe Färbung ändern.
14. Bestimmen Sie die optische Dichte bei 450 ± 10 nm mit einem Mikrotiterplatten-Reader **innerhalb von 15 Minuten**.

Weitere Angaben zum Test entnehmen Sie bitte der englischen Arbeitsanleitung.

Symbols / Symbole / Symbôles / Símbolos / Símbolos / Σύμβολα

REF	Cat.-No.: / Kat.-Nr.: / No.- Cat.: / Cat.-No.: / N.º Cat.: / N.–Cat.: / Αριθμός-Κατ.:
LOT	Lot-No.: / Chargen-Bez.: / No. Lot: / Lot-No.: / Lote N.º: / Lotto n.: / Αριθμός -Παραγωγή:
	Use by: / Verwendbar bis: / Utiliser à: / Usado por: / Usar até: / Da utilizzare entro: / Χρησιμοποιείται από:
	No. of Tests: / Kitgröße: / Nb. de Tests: / No. de Determ.: / N.º de Testes: / Quantità dei tests: / Αριθμός εξετάσεων:
CONC	Concentrate / Konzentrat / Concentré / Concentrar / Concentrado / Concentrato / Συμπύκνωμα
LYO	Lyophilized / Lyophilisat / Lyophilisé / Liofilizado / Liofilizado / Liofilizzato / Λυοφιλιασμένο
IVD	In Vitro Diagnostic Medical Device. / In-vitro-Diagnostikum. / Appareil Médical pour Diagnostics In Vitro. / Dispositivo Médico para Diagnóstico In Vitro. / Equipamento Médico de Diagnóstico In Vitro. / Dispositivo Medico Diagnostico In vitro. / Ιατρική συσκευή για In-Vitro Διάγνωση.
	Evaluation kit. / Nur für Leistungsbewertungszwecke. / Kit pour évaluation. / Juego de Reactivos para Evaluació. / Kit de avaliação. / Kit di evaluazione. / Κιτ Αξιολόγησης.
	Read instructions before use. / Arbeitsanleitung lesen. / Lire la fiche technique avant emploi. / Lea las instrucciones antes de usar. / Ler as instruções antes de usar. / Leggere le istruzioni prima dell'uso. / Διαβάστε τις οδηγίες πριν την χρήση.
	Keep away from heat or direct sun light. / Vor Hitze und direkter Sonneneinstrahlung schützen. / Garder à l'abri de la chaleur et de toute exposition lumineuse. / Manténgase alejado del calor o la luz solar directa. / Manter longe do calor ou luz solar directa. / Non esporre ai raggi solari. / Να φυλάσσεται μακριά από θερμότητα και άμεση επαφή με το φως του ηλίου.
	Store at: / Lagern bei: / Stocker à: / Almacene a: / Armazenar a: / Conservare a: / Αποθήκευση στους:
	Manufacturer: / Hersteller: / Fabricant: / Productor: / Fabricante: / Fabbricante: / Παραγωγός:
	Caution! / Vorsicht! / Attention! / ¡Precaución! / Cuidado! / Attenzione! / Προσοχή!

Symbols of the kit components see MATERIALS SUPPLIED.
Die Symbole der Komponenten sind im Kapitel KOMPONENTEN DES KITS beschrieben.
Voir MATERIEL FOURNI pour les symbôles des composants du kit.
Símbolos de los componentes del juego de reactivos, vea MATERIALES SUMINISTRADOS.
Para símbolos dos componentes do kit ver MATERIAIS FORNECIDOS.
Per i simboli dei componenti del kit si veda COMPONENTI DEL KIT.
Για τα σύμβολα των συστατικών του κιτ συμβουλευτείτε το ΠΑΡΕΧΟΜΕΝΑ ΥΛΙΚΑ.

IBL AFFILIATES WORLDWIDE

IBL International GmbH Flughafenstr. 52A, 22335 Hamburg, Germany	Tel.: E-MAIL: WEB:	+ 49 (0) 40 532891 -0 Fax: -11 IBL@IBL-International.com http://www.IBL-International.com
IBL International B.V. Zutphenseweg 55, 7418 AH Deventer, The Netherlands	Tel.: E-MAIL: WEB:	+ 49 (0) 40 532891 -0 Fax: -11 IBL@IBL-International.com http://www.IBL-International.com
IBL International Corp. 194 Wildcat Road, Toronto, Ontario M3J 2N5, Canada	Tel.: E-MAIL: WEB:	+1 (416) 645 -1703 Fax: -1704 Sales@IBL-International.com http://www.IBL-International.com

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