BIOGENIX
INC. PVT. LTD.

Free β-hCG ELISA

Enzyme Immunoassay for Quantitative Determination of Free Beta Subunit of Human Chorionic Gonadotropin in Human Serum

Instructions for use

1. SYMBOL LEGEND

IVD	In vitro diagnostic medical device	RCNS	Reconstitute with specified volume of liquid
CE	EC Declaration of conformity	EC REP	Authorized Representative in the European Community
REF	Catalogue number	LOT	Batch code
	Use by		Manufacturer
	Date of manufacture		Consult operating instructions
	Temperature limitation		Biological risks
	Contains sufficient for <n> tests	CONJ	Conjugate
MP	Coated microplate (96 wells)	SUB	Substrate
WASH P 20X	Wash solution, 20X concentrated	TRIAL 5000X	Trial, 5000X concentrated
CAL	Calibrators	ASSAYB	Assay buffer
CONTROL	Control	OD	Optical density
STOP	Stop solution	H2O	Deionized or distilled water
DIL	Sample diluent		Irritant
		Warning	

2. INTENDED USE

Free ß-hCG kit is provided for the **quantitative** determination of free beta subunit of human chorionic gonadotropin (ß-hCG) in human serum by solid phase enzyme immunoassay.

Heterodimeric hCG molecule consists of two subunits – alpha and beta. Beta subunit is unique, i.e. specific, only for hCG, in contrast to alpha subunit which is identical in luteinizing, follicle-stimulating and thyroid-stimulating hormones of human pituitary gland. Free ß-hCG can enter peripheral blood flow of pregnant women in two ways: either by secretion from trophoblast cells or as a result of hCG degradation.

Monitoring of serum ß-hCG level in pregnant women is widely used for prenatal detection of trisomy 21. During normal gestation ß-hCG concentration in serum gradually increases until 10^{th} week, and then begins to decrease. When the fetus is affected by Down's Syndrome, the level of free ß-hCG in maternal serum is increased.

Quantitative determination of free ß-hCG can also be used for diagnostics of trophoblastic disease and choriocarcynoma in women as well as some testicle malignancies in men. It is also used for patients monitoring with certain germinogenic tumors.

Prospective studies have reported that the combination of maternal serum free ß-hCG, PAPP-A (pregnancy associated plasma protein A), nuchal translucency of fetus, and evaluation of maternal age is the optimal method of prenatal screening for trisomy 21in first trimester of gestation.

This assay is not available for evaluation the risk of Trisomy 21 in the following countries: Austria, Belgium, Bulgaria, Czech Republic, Cyprus, Denmark, Germany, Estonia, Greece, Finland, Hungary, France, Ireland, Iceland,

Italy,Latvia, Liechtenstein, Lithuania, Luxemburg, Malta, Norway, Poland,Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, The Netherlands, United Kingdom.

3. PRINCIPLE OF TEST

Free ß-hCG is a “sandwich” type of solid-phase enzyme immunoassay, based on two monoclonal antibodies that are specific for different epitopes of free ß-hCG molecule. One of these antibodies is conjugated with horseradish peroxidase; the other is coated onto the inner surface of microwells.

The assay is performed in two steps. First, serum samples and assay buffer are added to the wells and incubated. During the first incubation free ß-hCG binds to immobilized antibody forming a complex. During the second incubation anti- free-ß-hCG antibody conjugated with horseradish peroxidase binds to the complex produced during the first incubation. Quantity of bound conjugate is directly proportional to the quantity of free ß-hCG in the serum sample (see Fig.1).

During incubation with substrate the colour is developing. The intensity of the colour correlates to the concentration of free ß-hCG in specimens or calibrators. Free ß-hCG concentration in the patient sample is read from a standard curve that is processed in each assay.

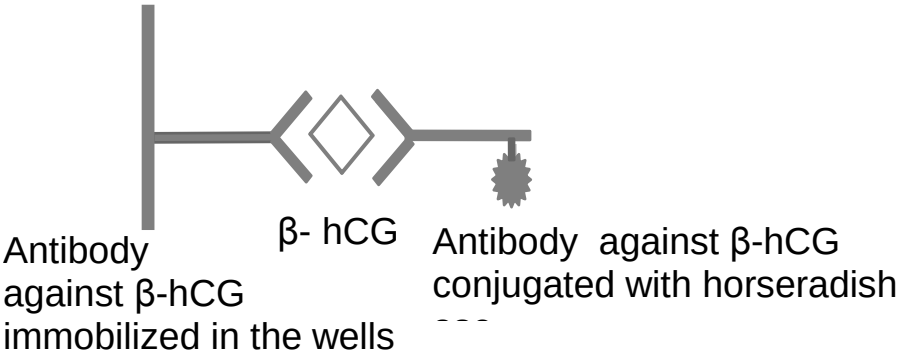

Fig. 1. Assay scheme

4. STORAGE AND STABILITY OF THE KIT

The expiration date of the kit is printed on the box label; the expiration date for each component is printed on the respective label.

Free ß-hCG kit should be stored at +2…+8 °C upon receipt, preferably in the original kit box, until the expiration date. Storage at +25 °C is allowed but for no more than 5 days.

Shelf life of the kit is 18 months.

After initial opening the kit is stable the expiration date if stored at +2…+8 °C.

If used in several separate experiments, after initial opening kit contents should be stored as follows:

- unused strips: in a firmly closed resealable zipper bag at +2…+8 °C until the expiration date;
- vials with conjugate, calibrators and controls (ready-to-use), sample diluent, substrate: at +2…+8 °C until the expiration date; vials with calibrators and controls (reconstituted): at +2…+8 °C for no more than 1 month after opening;
- vials with concentrated Trial, concentrated wash solution, assay buffer and stop solution: at +2…+8 °C until the expiration date;
- wash solution prepared for use: at room temperature (+18…+25 °C) for no more than 5 days or at +2…+8°C for no more 4 weeks, in a firmly closed bottle;
- Trial solution prepared for use: at room temperature (+18…+25 °C) for no more than 5 days, in a firmly closed bottle.

Damaged Test Kits

In case of any severe damage of the test kit or components, it has to be informed in writing, during one week after receiving the kit. Usage of severely damaged single components for a test run is not recommended.

5. SAMPLE COLLECTION AND STORAGE

Collect blood by venipuncture. After clotting, the serum is separated by centrifugation.

Do not use plasma, haemolyzed (bright red) or lipaemic (milky) serum samples as well as samples containing sodium azide as preservative.

Store serum samples at +2…+8 °C for no more than 5 days. Aliquot and freeze samples for longer storage (–20 °C and lower). Avoid repeated freezing.

6. EXPECTED VALUES

Serum samples from 863 pregnant women (at 9 to 13 weeks of gestation) were assayed with Free ß-hCG kit. Medians of ß-hCG concentration at different terms of gestation are given in Table 1.

Table 1

Term of gestation (full weeks)	Median concen-tration of free β-hCG (ng/mL)	Minimal/Maximal concentration of free β-hCG within the group (ng/mL)	Number of samples in the group
9	72.77	4.8/406.0	122
10	62.72	0.4/380.0	214
11	51.68	9.9/227.0	197
12	45.17	1.9/320.0	229
13	40.51	6.3/181.0	101

Median free β-hCG concentration for each term of pregnancy depends on different factors, e.g. on specificity of assay, some features of the screened population, accuracy of measurement in particular laboratory, etc.

For this reason it is highly recommended for each laboratory to determine reference ranges of median free ß-hCG concentrations for local population. Reference limits provided by the Manufacturer can be temporarily used as guidelines until such ranges are determined.

For risk prediction in prenatal screening results of free ß-hCG assay are presented as MOM (multiple of medians). MOM is calculated by the following formula:

MOM = Measured concentration of free ß-hCG/Median concentration of free β-hCG for the corresponding week of gestation.

It is recommended to use special software for automatic calculation of risk of Down Syndrome.

7. QUALITY CONTROL

It is recommended to use control samples according to the state and federal regulations. The use of control samples is advised to assure the day to day validity of results.

8. REAGENT PREPARATION

Allow all the reagents to reach room temperature, and then thoroughly stir.

MP Keep **microplate** at room temperature (+18…+25 °C) for at least 30 minutes before opening the bag. Place required number of strips into strip holder. Place unused strips onto the resealable zipper bag and reseal duly.

CAL **CONTROL** **Calibrators and Control**

Liquid control and calibrators are ready to use.

Prepare lyophilized calibrators and control as follows. Gently

tap on the vial caps to knock off all the dry matter. Open the vials and carefully place the caps upside down on the clean dry surface. Add 0.5 mL of distilled or deionized water to each vial with lyophilized calibrators and controls, close vials with the corresponding caps and leave for 10 min at room temperature without stirring. Then stir gently avoiding foaming, until the dry matter is completely dissolved. Leave for another 10 minutes at room temperature stirring gently periodically. Make sure that no dry matter is left on the caps and walls of the vials.

WASH P Prepare required volume of **wash solution** by dilution of the concentrate 20-fold with distilled or deionized water. For example:

5 mL of **WASH P** **20X** + 95 mL of water.

Mix thoroughly, avoid foaming.

SUB Protect **substrate** from direct light.

9. SAMPLE PREPARATION

Allow samples to reach room temperature. Stir samples gently in order to ensure homogeneity.

If expected ß-hCG concentration in the samples is higher than in **calibrator 5** **CAL5**, the samples should be diluted 5-fold with sample **Calibrator 0** **CAL0** or **DIL** in concordance with instructions for use before analysis. The example of manual sample dilution as follows:

40 µL of **CAL0** + 10 µL of serum sample

10. PERFORMANCE CHARACTERISTICS OF THE ASSAY

10.1. Calibration - Traceability

Free ß-hCG test kit was calibrated against the WHO International Standard IRP 75/551.

10.2. Analytical Sensitivity

Analytical sensitivity of **Free ß-hCG kit** i.e. concentration that can be distinguished from zero calibrator, is 2 ng/mL. It is defined as mean OD of 10 replicates of calibrator 0 plus 2SD.

10.3. Dilution Parallelism of Serum Samples

Serial dilutions of two human serum samples with predetermined free ß-hCG concentration in calibrator 0 were assayed with **Free ß-hCG kit** with the following result:

Sample	Dilution	Expected concentration, ng/mL	Measured concentration, ng/mL	Measured/expected concentration ratio, %
1	Undiluted		20.81	
	1:2	10.41	10.88	104.6
	1:4	5.20	5.01	96.3
	1:8	2.60	2.51	96.5
2	Undiluted		154.27	
	1:2	77.14	71.21	92.3
	1:4	38.57	39.53	102.5
	1:8	19.28	20.78	107.8

10.4. Specificity

According to Product specification, provided by the supplier, cross-reactivity of both monoclonal antibodies with LH, TSH and FSH does not exceed 1.0 %.

10.5. Measurement Range

Free ß-hCG kit was validated for measurement of ß-hCG concentration within the concentration diapason (without dilution) of 2 – 200 ng/mL.

10.6. Intra- and Inter-Assay Variation (Precision)

Intra-assay CV

For **intra-assay CV** determination 8 serum samples were run, each in 9 replicates. The results are shown below.

Sample	Mean free ß hCG concentration, ng/mL			Inter-assay precision	
	1 assay	2 assay	3 assay	SD	CV, %
HS 1	15.0	17.3	16.0	1.15	7.2
HS 2	28.7	32.7	30.4	2.01	6.6
HS 3	44.9	50.5	46.6	2.87	6.1
HS 4	75.1	86.1	80.0	5.51	6.9
HS 5	92.3	104.8	96.5	6.36	6.5
HS 6	107.5	125.6	112.9	9.29	8.1
HS 7	116.9	133.9	122.3	8.69	7.0
HS 8	164.0	183.2	159.0	12.78	7.6

Inter-assay CV

For inter-assay CV determination, 8 serum samples were assayed 9 times by different operators with 1-week interval. Each specimen was run in 2 replicates. The results are shown below.

Sample	Mean free ß hCG concentration, ng/mL	Intra-assay CV	
		SD	CV, %
HS 1	27.5	1.89	6.9
HS 2	46.4	2.64	5.7
HS 3	59.7	2.51	4.2
HS 4	61.8	3.58	5.8
HS 5	105.8	3.07	2.9
HS 6	127.6	6.76	5.3
HS 7	151.4	10.45	6.9
HS 8	182.9	13.35	7.3

HS 2	34.1	32.3	32.5	0.95	2.9
HS 3	57.9	64.6	58.7	3.65	6.0
HS 4	75.2	86.0	81.2	5.43	6.7
HS 5	110.6	119.7	106.5	6.75	6.0
HS 6	128.6	128.9	127.9	0.52	0.4
HS 7	155.9	161.9	146.9	7.52	4.9
HS 8	190.3	193.3	173.1	10.89	5.9

11. LIMITATION OF THE METHOD

Any clinical diagnosis should not be based on the results of in vitro diagnostic method alone. For diagnosis establishment, a physician is supposed to consider all available clinical and laboratory findings.

12. SAFETY PRECAUTIONS

- **This kit is for in vitro diagnostic use only.** Operator should follow the manual closely in order to ensure reliable data. The manual is valid for the present kit only, within the listed composition. Any substitution of kit components is not allowed by CE regulations.
- Do not use kit or its components after the expiration date indicated on the label. Take into account stability period for reconstituted reagents.
- Do not mix or use together reagents from different lots of the kit except substrate, stop solution and wash solution.
- Do not use substrate, stop solution and wash solution supplied by other vendors.
- Use only “P”-labeled wash solution.
- Note that stop solution is 1 N HCl solution. Avoid contacts with skin and mucosa. In case of contact rinse affected area thoroughly with plenty of water and seek medical advice.

- Take into account the following common procedural notes:

  -always pipette reagents into wells immediately after washing procedure;

  -avoid contamination of the solutions;

  -in case of partial use of the kit, dispense only required volume of the reagent into the tray;

  -do not pour unused reagents back into the original vials;

  -avoid exposure to direct sunlight during incubations;

  -always pipette reagents in the same order to minimize reaction time differences between wells; the total dispensing time for the calibrators, control and samples must not exceed 15 min;

  -the incubation temperature for all the immunological reactions must be kept at +37 °C;

  -do not touch the bottom of the wells;

  -calibrators should be measured in each separate assay. It is also recommended to measure each time ß-hCG concentration in the control.

- TMB solution should be colourless. Light colouring of solution is admissible. Avoid direct exposure of substrate to sunlight.

-  Source materials of human origin used for kit components preparation were tested and found negative for HBsAg, anti-HIV and anti-HCV antibodies. However, none of known laboratory test guarantees absence of these viral agents. Therefore, all kit components and patient's samples should be handled as potentially hazardous.

-  After usage strips, calibrators, controls, specimens and all consumables which contacted with specimens during handling, storage or assay (tubes, vials, gloves, pipette tips etc.) should be collected separately and sterilized by autoclaving. Instead of autoclaving pipette tips may be sterilized by disinfectant treatment. After sterilization all components and expendable materials may be utilized as non-dangerous garbage. Other components of the kit should be discarded into conventional garbage.

-  During manual washing procedure do not discard the contents of the wells directly to drainage. Use a container with disinfectant solution.

-  As the kit contains potentially hazardous material, the following precautions should be taken:

  -do not smoke, eat or drink while performing the assay;

  -always use protective gloves;

  -never pipette material by mouth;

  -in case of spilling, wipe up the spills promptly and wash affected area thoroughly using decontaminant.

- GLP including all general and individual regulations should be applied for the kit usage.

 As the kit contains irritant (CONJ, CAL, DIL, CONTROL), the following precautions should be observed:

- P261 - Avoid breathing spray;
- P272 - Contaminated work clothing should not be allowed out of the workplace;

- P280 - Wear protective gloves/protective clothing/eye protection;
- P302+P352 - IF ON SKIN: Wash with plenty of soap and water;
- P333+P313 - If skin irritation or rash occurs: Get medical advice/attention;
- P363 - Wash contaminated clothing before reuse;
- P501 - Dispose of contents/container in accordance with national regulation.

Precautionary statements according to Regulation EC № 1272/2008.

13. MATERIAL PROVIDED

13.1. Material Provider

MP	**Microplate:** 12 breakable 8-well strips (total 96 wells) coated with anti-ß-hCG monoclonal antibodies	1 pcs
CONJ	**Conjugate:** solution contains anti- free ß-hCG monoclonal antibodies conjugated with HRP	14 mL, ready to use
0–5 CAL	free **ß-hCG calibrators:** protein-based solution or lyophilized preparations containing known ß-hCG concentrations - 0; 8; 25; 50; 100; 200 ng/mL. The concentrations of calibrators may be different for schemes with or without shaking. For exact ß-hCG concentrations, see vial labels.	6 vials, 0.5 mL each; ready to use or lyophilized preparations
CONTROL	free **ß-hCG control:** protein-based solution or lyophilized preparation containing known free ß-hCG concentration. The range of ß-hCG concentration may be different for schemes with or without shaking. For exact range of ß-hCG concentration see vial label.	0.5 mL, ready to use or lyophilized preparation
WASH P 20X	**Wash solution P, 20X concentrated:** surfactant in buffered saline, sufficient for preparation of 840 mL of solution	3 vials, 14 mL each, concentrated
SUB	**Substrate (TMB solution):** 3,3',5,5'-tetramethylbenzidine solution in citrate buffer containing hydrogen peroxide	14 mL, ready to use

STOP	**Stop solution:** 1 N HCl solution	14 mL, ready to use
ASSAYB	**Assay buffer**	25 mL, ready to use

13.2. Equipment and Materials Required but not provided

- 1-channel calibrated variable precision pipettes, with disposable tips;
- 8-channel calibrated variable precision pipette, with disposable tips;
- microplate incubator (+37 °C) or microplate incubator/shaker (37°C, shaking speed 500–800 rpm);
- manual or automatic equipment for rinsing wells;
- calibrated microplate reader (450 nm);
- vortex tube mixer;
- deionized or distilled water;
- graduated beaker and cylinder of appropriate volume;
- latex or plastic gloves;
- trays for pipetting reagents with 8-channel pipette;
- disinfectant;
- absorbent material (for manual wash).

13.3. Test Procedure

Free ß-hCG kit is designed for 96 tests. This is sufficient for 40 unknowns, 6 calibrators, 1 control and 1 blank (OD of TMB solution) in duplicates, provided that all the strips are used simultaneously.

13.3.1. Assay Procedure

13.3.1.1. (Protocol with shaking

(See assay scheme, section 13.5.)

All samples should be tested in duplicates.

A. Pipette**:**

- **200 µL** of Assay buffer **ASSAYB** into each well,

Leave wells A1-A2 empty for blank!

B. Pipette**:**

- **20 µL** of calibrators **CAL**, control **CONTROL** and patients' samples in duplicates**;**

except wells A1-A2.

Note: total time of dispensing must not exceed 15 minutes, otherwise the test result may be unreliable, because the time of incubation will substantially vary for different samples

C. Incubate for **45 minutes at 37°C while shaking (500–800 rpm)**.

D. Wash 5 times, as described in section 13.3.2.

E. Pipette**:**

- **100 µL** of conjugate **CONJ** into each well,

Leave wells A1-A2 empty for blank!

F. Incubate for **15 minutes at 37°C without shaking**.

G. Wash 5 times, as described in section 13.3.2.

H. Pipette **100 µL** of **substrate SUB** into each well (including blank); incubate strips **at room temperature** (18-25 °C) **in the dark for 15-30 minutes**, depending on the colour intensity, or **10 minutes while shaking (500-800 rpm) at 37 °C**.

I. Pipette **100 µL** of **stop solution STOP** to all the wells in the same sequence and at the same speed as used for dispensing TMB substrate. Shake for 1–2 min at room temperature.

J. Read OD at **450 nm within 20 min**.

13.3.1.2. Protocol without shaking

(See assay scheme, section 13.6.)

All samples should be tested in duplicates.

A. Pipette**:**

- **200 µL** of Assay buffer **ASSAYB** into each well,

Leave wells A1-A2 empty for blank!

B. Pipette**:**

- **20 µL** of calibrators **CAL**, control **CONTROL** and patients' samples in duplicates**;**

except wells A1-A2.

Note: total time of dispensing must not exceed 15 minutes, otherwise the test result may be unreliable, because the time of incubation will substantially vary for different samples

C. Incubate for 60 minutes at 37°C (Pre-shake for 1-2 min at room temperature).

D. Wash 5 times, as described in section 13.3.2.

E. Pipette**:**

- **100 µL** of conjugate **CONJ** into each well,

Leave wells A1-A2 empty for blank!

F. Incubate for **15 minutes at 37°C**.

G. Wash 5 times, as described in section 13.3.2.

H. Pipette **100 µL** of **substrate SUB** into each well (including blank); incubate strips **at room temperature** (18-25 °C) **in the dark for 15-30 minutes**, depending on the colour intensity.

I. Pipette **100 µL** of **stop solution STOP** to all the wells in the same sequence and at the same speed as used for dispensing TMB substrate. Shake for 1–2 min at room temperature.

J. Read OD at **450 nm within 20 min**.

13.3.2. Wash Procedure

It is advisable to use an automatic microplate washer set at 5 wash cycles and a volume of 300 µL of wash solution per well per cycle.

If an automatic washer is not available, the wash procedure can be carried out manually as follows:

- remove the contents of the wells into a container with disinfectant;
- dispense 300 µL of wash solution, prepared according to section 8., into each well, shake the plate carefully for 5–10 sec and remove the contents of the wells; repeat 5 times;
- strike the wells sharply on absorbent material to remove any liquid residue.

13.4. Data Processing

If the reader cannot be adjusted to zero using the substrate blank in wells A1-A2, subtract mean OD value of wells A1-A2 from all OD values before further calculations.

Example:

OD (Cal 5) measured = 2.28 and OD (blank) = 0.06;

OD (Cal 5) calculated = 2.28 – 0.06 = 2.22

13.4.1. Data Reliability (for OD Measured at 450 nm)

The data should meet the following criteria:

- average blank OD (in wells A1-A2) ≤0.100;
- average OD of Cal 5 ≥1.5 (after blank subtraction);
- control’s concentration must fall within the acceptability range that are shown on the vial label.

If the data obtained do not meet the criteria, the results are considered unreliable and the test should be repeated.

13.4.2. Quantitative Determination

Specialized software for quantitative determination is recommended. Mean OD values of the calibrators at 450 nm versus their respective ß-hCG concentrations using 4PL or 5PL fit (see typical standard curve, fig. 2). Calculate concentration of ß-hCG in samples using standard curve.

Any extrapolation of the standard curve to free ß-hCG concentration above the nominal value of calibrator 5 is forbidden. In this case the sample should be diluted 5-fold with Calibrator 0 and re-tested. Multiply the measured concentration of pre-diluted samples by dilution factor (5-fold).

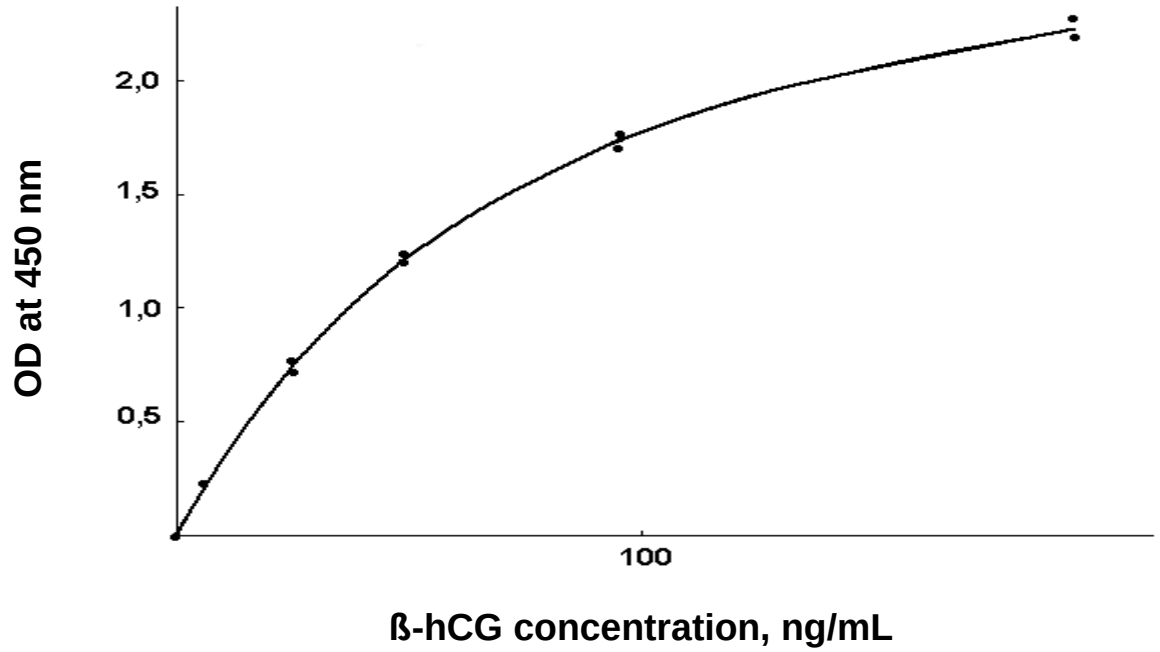

Fig. 2. Example of typical standard curve.
Do not use for evaluation of real assay data!

13.5. Assay scheme with shaking

Wells / Reagents	«Blank»	**CAL** **CONTROL**	Samples
ASSAYB	–	200 µL	200 µL
CAL **CONTROL**	–	20 µL	–
Samples	–	–	20 µL
Incubation №1	45 min, 37 °C, 500–800 rpm		
WASH **P** (diluted)	5 x 300 µL		
CONJ	–	100 µL	100 µL
Incubation №2	15 min, 37 °C, without shaiking		
WASH **P** (diluted)	5 x 300 µL		
SUB	100 µL	100 µL	100 µL
Incubation №3	15–30 min, 18-25 °C, in the dark		
	10 min, 37°C, 500–800 rpm		
STOP	100 µL	100 µL	100 µL
Stirring	1–2 min, 18-25 °C		
OD measuring	450 nm		
Calculations	Corresponding software		

13.6. Assay scheme without shaking

Wells / Reagents	«Blank»	**CAL** **CONTROL**	Samples
ASSAYB	–	200 µL	200 µL
CAL **CONTROL**	–	20 µL	–
Samples	–	–	20 µL
Incubation №1	60 min, 37 °C (Pre-shake for 1-2 min at room temperature)		
WASH P (diluted)	5 x 300 µL		
CONJ	–	100 µL	100 µL
Incubation №2	15 min, 37 °C		
WASH P (diluted)	5 x 300 µL		
SUB	100 µL	100 µL	100 µL
Incubation №3	15–30 min, 18-25 °C, in the dark		
STOP	100 µL	100 µL	100 µL
Stirring	1–2 min, 18-25 °C		
OD measuring	450 nm		
Calculations	Corresponding software		

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