

**ENZYME IMMUNOASSAY FOR THE QUANTITATIVE
DETERMINATION OF HUMAN CHORIONIC
GONADOTROPIN (HCG) IN SERUM**

FOR IN VITRO DIAGNOSTIC USE

Store at 2 to 8°C.

INTENDED USE

The hCG ELISA is intended for the quantitative determination of human chorionic gonadotropin (hCG) concentration in human serum. The assay is for use in the early diagnosis of pregnancy in a clinical laboratory setting.

INTRODUCTION

Human chorionic gonadotropin (hCG) is a glycoprotein hormone normally produced by the placenta during pregnancy.¹⁻³ The hCG molecule consists of two combined, dissimilar subunits designated alpha and beta. The beta subunit, with a molecular weight of approximately 30,000 daltons, confers biological and immunological specificity to the entire hCG molecule by virtue of its unique amino acid sequence and content. The alpha subunit, with a molecular weight of approximately 18,000 daltons, is essentially identical to the alpha subunit of the pituitary glycoprotein hormones: luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH).²⁻¹⁰

The appearance of hCG in urine or serum soon after conception and its rapid rise in concentration makes it an ideal indicator for the detection and confirmation of pregnancy. However, elevated hCG levels are also frequently associated with trophoblastic and non-trophoblastic neoplasms; these conditions should be considered before a diagnosis of pregnancy can be made.^{11-17, 31-35}

In women with a multiple pregnancy (twins, triplets, etc.), levels of hCG have been reported to be higher than those expected during a normal single pregnancy. This is probably the result of the increased placental mass necessary to sustain multiple fetuses. Also, as one might suspect, cases of placental insufficiency show levels of hCG lower than those expected during normal pregnancy. Decreased values have also been associated with threatened abortion and ectopic pregnancy.²⁵⁻³⁰

Immunoassays utilizing antibodies specific to the beta subunit of hCG provide a sensitive and specific technique allowing early detection of pregnancy around the time of the first missed menstrual period.¹⁸⁻²⁴

PRINCIPLE OF THE ASSAY

The hCG ELISA Test is based on a solid phase enzyme-linked immunosorbent assays (ELISA).^{26, 27} The assay system utilizes one mouse monoclonal anti-hCG antibody for solid phase (microtiter wells) immobilization and another mouse monoclonal anti-hCG antibody in the antibody-enzyme (horseradish peroxidase) conjugate solution.

The test specimen (serum) is added to the hCG antibody coated microtiter wells and incubated with the Zero Buffer at room temperature for 30 minutes. If hCG is present in the specimen, it will combine with the antibody on the well. The well is then washed to remove any residual test specimen, and hCG antibody labeled with horseradish peroxidase (conjugate) is added. The conjugate will bind immunologically to the hCG on the well, resulting in the hCG molecules being sandwiched between the solid phase and enzyme-linked antibodies.

After incubation at room temperature for 15 minutes, the wells are washed with water to remove unbound-labeled antibodies. A solution of TMB is added and incubated at room temperature for 20 minutes, resulting in the development of a blue color. The color development is stopped with the addition of 1N HCl, and the color is changed to yellow and measured spectrophotometrically at 450 nm. The concentration of hCG is directly proportional to the color intensity of the test sample.

REAGENTS AND MATERIALS PROVIDED

1. Antibody-Coated Wells (1 plate, 96 wells)
Microtiter wells coated with mouse monoclonal anti-hCG.
2. hCG Zero Buffer (1 dropper vial, 13 mL)
Contains bovine serum, green dye, Na₂S₂O₃.
3. Enzyme Conjugate Reagent (18 mL)
Contains mouse monoclonal anti-hCG (specific to hCG, β -hCG) conjugated to horseradish peroxidase.
4. Reference Standard Set (1 mL/vial)
Contains 0, 5, 20, 50, 150 and 300 mIU/mL (WHO, 1st IRP/3rd IS, 75/537) human chorionic gonadotropin in bovine serum with Na₂S₂O₃.
5. TMB Reagent (1 bottle, 11 mL)
Contains 3, 3', 5, 5' tetramethylbenzidine (TMB) stabilized in buffer solution.
6. Stop Solution (1N HCl) (1 bottle, 11 mL)
Contains diluted hydrochloric acid.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Distilled or deionized water
2. Precision pipettes: 0.05, 0.1, 0.15, 0.20 and 1 mL
3. Disposable pipette tips
4. Microtiter well reader capable of reading absorbance at 450nm.
5. Vortex mixer, or equivalent
6. Absorbent paper
7. Graph paper
8. Quality control material (e.g., BioRad Lyphochek Control sera)

WARNINGS AND PRECAUTIONS

1. **CAUTION:** This kit contains human material. The source material used for manufacture of this kit tested negative for

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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 and disposal should be as defined by an appropriate national biohazard safety guideline or regulation, where it exists.²¹

2. Do not use reagents after expiration date and do not mix or use components from kits with different lot numbers.
3. Do not use the reagent when it becomes cloudy or contamination is suspected.
4. Do not use the reagent if the vial is damaged.
5. Replace caps on reagents immediately. Do not switch caps.
6. Each well can be used only once.
7. Do not pipette reagents by mouth.
8. Solutions containing additives or preservatives, such as sodium azide, should not be used in the enzyme reaction.
9. 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.
10. For in vitro diagnostic use.

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. The opened and used reagents are stable until the expiration date if stored properly at 2-8°C.
3. Keep microtiter plate in a sealed bag with desiccant to minimize exposure to damp air.

SPECIMEN COLLECTION AND PREPARATION

1. 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. Avoid grossly hemolytic (bright red), lipemic (milky), or turbid samples.
2. Specimens should be capped and may be stored for up to 48 hours at 2-8°C. Specimens held for a longer time should be frozen only once at -20°C prior to assay. Thawed samples should be inverted several times prior to testing.

INSTRUMENTATION

A microtiter well reader with a bandwidth of 10 nm or less and an optical density range of 0 to 2 OD or greater at 450 nm wavelength is acceptable for absorbance measurement.

REAGENT PREPARATION

1. All reagents should be allowed to reach room temperature (18-25°C) before use.
2. All reagents should be mixed by gentle inversion or swirling prior to use. Do not induce foaming.
3. Samples with expected values greater than 300 mIU/mL should be diluted with Zero Standard or normal human male serum (1:100 initial dilution) prior to assaying.

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 50 µL of standards, samples, and controls into appropriate wells.
3. Gently mix for 30 seconds.
4. Dispense 100 µL of hCG Zero Buffer into each well.
5. Thoroughly mix for 10 seconds. It is very important to have complete mixing.
6. Incubate at room temperature (18-25°C) for 30 minutes.
7. Remove the incubation mixture by flicking plate contents into a suitable waste container.
8. Rinse and flick the microtiter wells 5 times with distilled or deionized water. (Please do not use tap water.)
9. Strike the wells sharply onto absorbent paper or paper towels to remove all residual water droplets.
10. Dispense 150 µL of Enzyme Conjugate Reagent into each well. Gently mix for 5 seconds.
11. Incubate at room temperature for 15 minutes.
12. Remove the incubation mixture by flicking well contents into a suitable waste container.
13. Rinse the wells 5 times with distilled or dionized water. (Please do not use tap water.)
14. Strike the wells sharply on absorbent paper to remove residual water droplets.
15. Dispense 100 µl TMB Reagent into each well. Gently mix for 5 seconds.
16. Incubate at room temperature in the dark for 20 minutes.
17. Stop the reaction by adding 100 µL of Stop Solution into each well.
18. Gently mix for 5 seconds.
19. Read OD at 450 nm with a microtiter well reader within 15 minutes.

CALCULATION OF RESULTS

1. Calculate the mean absorbance value (OD₄₅₀) for each set of reference standards, controls and samples.
2. Construct a standard curve on linear graph paper, by plotting the mean absorbance obtained for each reference standard against its concentration in mIU/mL, with absorbance on the vertical (y) axis and concentration on the horizontal (x) axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of hCG in mIU/mL from the standard curve. Depending on experience and/or the availability of computer capability, other methods of data reduction may be employed.
4. If patient sample has a hCG concentration greater than 300 mIU/mL, please dilute 100 fold with the Zero Standard or normal male human serum and re-test (0.01 mL sample plus 0.99 mL Zero).

5. Any diluted samples must be further corrected by the appropriate dilution factor.

PROCEDURAL NOTES

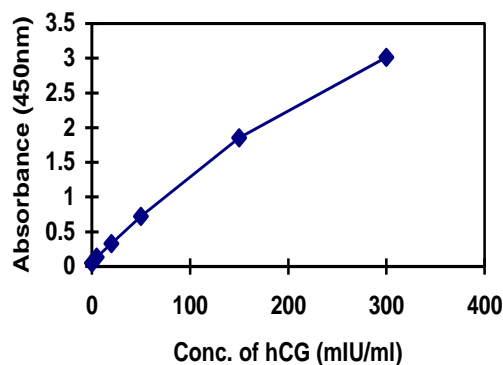
1. Manual Pipetting: It is recommended that no more than 32 wells be used for each assay run. Pipetting of all standards, samples, and controls should be completed within 3 minutes.
2. Automated Pipetting: A full plate of 96 wells may be used in each assay run. However, it is recommended that pipetting of all standards, samples, and controls be completed within 3 minutes.
3. All standards, samples, and controls should be run in duplicate concurrently so that all conditions of testing are the same.
4. It is recommended that the wells be read within 15 minutes following addition of Stop Solution.

INTERPRETATION OF RESULTS

1. Results of a typical standard run of the assay are shown below.

hCG (mIU/mL)	Absorbance (450nm)
0	0.051
5	0.134
20	0.328
50	0.724
150	1.852
300	3.011

2. This standard curve is for illustration only, and should not be used to calculate unknowns.



EXPECTED VALUES

Each laboratory must establish its own normal range based on patient population.

hCG is not normally detected in the serum of healthy men or healthy non-pregnant women. The concentration of hCG in the serum of pregnant women increases to 5-50 mIU/mL one week after implantation and continues increasing exponentially during the first ten weeks, reaching a maximum of 100,000 – 200,000 mIU/mL at the end of the first trimester.^{9,19,30,31}

Although normal pregnancy is usually the cause of increased hCG levels in urine and serum, elevated hCG concentrations have also been reported in patients diagnosed with molar pregnancy, chorio-carcinoma, and non-trophoblastic neoplasms.¹²⁻¹⁸

PERFORMANCE CHARACTERISTICS

1. Accuracy

A statistical study using 53 healthy patient samples (4 non-pregnant, 49 pregnant) ranging in hCG concentration from 2.4 mIU/mL to 948 mIU/mL, demonstrated good correlation with two commercially available kits as shown below.

- a. Comparison between this hCG Immunoassay Test Kits and the BioRad CoTube™ hCG IRMA kits provided the following data:

N = 53
 Correlation coefficient = 0.994
 Slope = 0.992
 Intercept = -0.781
 Mean = 267.5 mIU/mL
 Bio-Rad Mean = 264.5 mIU/mL

- b. Comparison between this kit and the Serono hCG MAIAclone kits provided the following data:

N = 53
 Correlation coefficient = 0.996
 Slope = 0.902
 Intercept = 4.970
 Mean = 267.5 mIU/mL
 Serono Mean = 246.2 mIU/mL

2. Sensitivity

The minimum detectable concentration of the hCG ELISA assay as measured by 2SD from the mean of a zero standard is estimated to be at least 2.0 mIU/mL.

3. Precision

- a. *Intra-Assay Precision*

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

Serum Sample	1	2	3
Number of Replicates	24	24	24
Mean hCG (mIU/mL)	4.8	18.8	138.1
Standard Deviation	0.26	0.61	4.61
Coefficient of Variation (%)	5.5%	3.3%	3.3%

- b. *Inter-Assay Precision*

Between-run precision was determined by replicate measurements of three different serum samples over a

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series of individually calibrated assays. Between-assay variability is shown below:

Serum Sample	1	2	3
Number of Replicates	30	30	30
Mean hCG (mIU/mL)	5.1	19.1	146.5
Standard Deviation	0.36	0.74	6.04
Coefficient of Variation (%)	6.9%	3.9%	4.1%

4. Recovery and Linearity Studies

a. Recovery

Various patient serum samples of known hCG levels were combined and assayed in duplicate. The mean recovery was 96.2%.

	Expected Concentration (mIU/mL)	Observed Concentration (mIU/mL)	% Recovery
	283.9	271.9	95.8%
	147.1	148.3	100.8%
	78.1	72.7	93.1%
	38.4	37.0	96.4%
	20.3	19.5	96.1%
	9.8	9.0	91.8%
Mean: 95.7%			
	249.1	257.5	103.4%
	135.1	134.1	99.3%
	70.6	67.9	96.2%
	37.8	33.8	89.4%
	19.0	17.8	93.7%
	8.7	8.5	97.7%
Mean: 96.6%			

b. Linearity

Three patient samples were serially diluted to determine linearity. The mean recovery was 109.1%.

#	Dilution	Expected Conc. (mIU/ml)	Observed Conc. (mIU/ml)	% Expected
1.	Undiluted	--	110.0	--
	1:2	55.0	54.8	99.6%
	1:4	27.5	29.8	108.4%
	1:8	13.8	15.0	108.7%
	1:16	6.9	7.5	108.7%
	1:32	3.5	4.0	114.3%
Mean = 107.9%				
2.	Undiluted	--	323.1	--
	1:2	161.6	1.67	103.9%
	1:4	80.8	85.8	106.2%
	1:8	40.4	44.2	109.4%
	1:16	20.2	23.6	116.8%
	1:32	10.1	11.3	116.9%
	1:64	5.1	6.1	119.6%
Mean = 111.3%				

3.	Undiluted	----	840	----
	1:2	420	445	106.0%
	1:4	210	219	104.3%
	1:8	105	113	107.6%
	1:16	52.5	57.8	110.1%
	1:32	26.3	28.9	109.9%
	1:64	13.2	14.7	111.4%
Mean = 108.2%				

5. Hook Effect

No high dose hook effect is observed in this assay at hCG levels up to 1,000,000 mIU/mL.

6. Specificity

The following hormones were tested for cross-reactivity:

HORMONE TESTED	CONCENTRATION	READ AS TOTAL HCG IN SERUM (mIU/mL)
Beta-hCG	10 ng/ml	14.8
	50 ng/ml	65.1
	100 ng/ml	111.7
	250 ng/ml	197.6
Alpha-hCG	10 ng/ml	2.1
	50 ng/ml	13.9
	100 ng/ml	26.8
	250 ng/ml	55.9
LH (WHO, 1st IRP 68/40)	25 mIU/ml	0.0
	50 mIU/ml	0.2
	100 mIU/ml	1.1
	250 mIU/ml	4.3
	500 mIU/ml	8.0
TSH (WHO, 2nd IRP 80/558)	25 µIU/ml	0.0
	100 µIU/ml	0.0
	250 µIU/ml	0.0
FSH (WHO, 2nd IRP-HMG)	50 mIU/ml	0.0
	250 mIU/ml	0.0
	500 mIU/ml	0.0
HGH (WHO, 1st IRP 65/217)	25 ng/ml	0.0
	50 ng/ml	0.0
	100 ng/ml	0.0
Prolactin (WHO, 1st IRP 75/504)	50 ng/ml	0.0
	250 ng/ml	0.0
	500 ng/ml	0.0

STANDARDIZATION

The Reference Standards are calibrated against the World Health Organization's (WHO) First IRP (75/537) and 3rd International Standard (IS) (75/537). The hCG TEST Kit measures the intact hCG plus the beta subunit. Our data shows that 1st IRP and 3rd IS reference standards yield statistically equivalent results.

From 1964 until early in 1982, the 2nd IS was used for reporting bioassay and immunoassay values. Biologically active hCG

molecules comprise only approximately 20% of this standard. In 1974 the WHO Expert Committee on Biological Standardization established the 1st IRP (75/537) of highly purified, biologically active hCG.

Interpretation of results, especially when correlating kits from different manufacturers, should be made with the possible differences in standardization in mind. Commercial assays calibrated to the 1st IRP (75/537) and 3rd IS (75/537) may give results twice the value of assays calibrated using the 2nd IS.

The difference in interpretation of commercial reference standards is due to the variability of assay systems employed and the different hCG antibodies used in the hCG Test Kit and other commercial kits. The lower values obtained with the 2nd IS are due to the impure nature of this standard, which contains large amounts of disassociated alpha and beta subunits in addition to intact hCG molecules.

QUALITY CONTROL

Good laboratory practice requires that quality control specimens (controls) be run with each calibration curve to verify assay performance. To assure proper performance, a statistically significant number of controls should be assayed to establish mean values and acceptable ranges.

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 with adherence to good laboratory practice.
2. This product is not intended to be used for the risk calculation of trisomy 21.
3. 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.
4. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
5. A normal pregnancy cannot be distinguished from an ectopic pregnancy based on the hCG level alone. Also, spontaneous miscarriage may cause confusion in interpreting test results.

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