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Review

Immunoassay of human chorionic gonadotropin, its free subunits, and metabolites

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Multiple hCG-related molecules are present in pregnancy serum and urine samples. These include nonnicked hCG (the hormone), nicked hCG, hyper- and hypoglycosylated hCG, hCG missing the C-terminal extension, free α-subunit, large free α-subunit, free β-subunit, nicked free β-subunit, and β-core fragment. Over 100 immunoassays are sold for quantifying hCG-related molecules in serum or urine. Each measures nonnicked hCG and one of seven combinations of the other hCG-related molecules. This is the source of interassay discordance in hCG determinations. Whereas minor variations are noted in different kit results in normal pregnancy samples (more than twofold variation), much larger variations may be found in two immunoassay results in irregular gestations (spontaneous abortion, aneuploidy, preeclampsia, cancers, and trophoblast disease). Care is needed in choosing an immunoassay. What the assay measures may be more important than its cost or speed. This article reviews the structure of hCG and related molecules. It examines the stability and degradation of hCG, and recognition of hCG-related molecules by different types of immunoassay. Also reviewed are new assays for specifically detecting these other hCG-related molecules.

Multiple human chorionic gonadotropin (hCG)-related molecules are present in pregnancy serum and urine samples.[1] These include degraded hCG molecules, hyper- and hypoglycosylated hCG, free subunits, large free subunits, and fragments. There are >100 commercial assays available for measuring hCG concentrations in serum and urine samples. Each uses any of seven common antibody combinations. Some of these antibody combinations may detect only undamaged or nonnicked hCG molecules, some require the C-terminal segment of the β-subunit to be intact, some detect nonnicked molecules and free β-subunit (free β), others detect nicked and nonnicked hCG molecules, and still others detect nicked and nonnicked hCG molecules plus free β. Only a few of the assays detect β-core fragment, the principal form of hCG β-subunit in urine samples. The multiple combinations of antibodies used in commercial assays today are a cause of heterogeneity. In extreme cases, interassay heterogeneity can cause as much as 50-fold difference in hCG immunoassay results. In certain instances, like after clearance of hCG after termination, after trophoblast disease, or examining persistent low concentrations of hCG, interassay discordance may lead to false-positive or false-negative hCG results. Interassay discordance should be of great importance to clinical and research chemists setting up or running an hCG immunoassay program.

This review article examines the presence of nicked and otherwise degraded hCG molecules, free subunits, and fragments in normal and abnormal pregnancies, and their effect on the hCG immunoassay. I start by examining the structure and metabolism of hCG; the stability of hCG, free subunits, and metabolites in samples; and the effect of the molecular heterogeneity of hCG on the immunodiagnosis of pregnancy. Particular problems with hCG measurement are addressed. Potential problems with detecting hCG in aneuploid pregnancies, trophoblast disease, and cancer are discussed. The difficulty measuring clearing concentrations of hCG and the interpretation of persistent low concentrations of hormone are elucidated. The different hCG immunoassay calibrators are described. New commercial assays are described for specifically measuring degraded or dissociated hCG molecules, and potential clinical applications are discussed.

Structure and Metabolism of hCG

hCG is a glycoprotein hormone composed of two dissimilar subunits, α and β, joined noncovalently. It is produced by trophoblast tissue in pregnancy and trophoblast disease, and in small amounts by certain poorly differentiated cancers. The α-subunit of hCG is similar to that of the pituitary glycoprotein hormones. It is composed of 92 amino acids linked by five disulfide bridges. The α-

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[1] Nonstandard abbreviations: hCG, human chorionic gonadotropin; free β, free β-subunit; free α, free α-subunit; and GnRH, gonadoliberin.

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subunit has two N-linked oligosaccharide side chains, attached at amino acid residues 52 and 78. The β-subunit is unique, and distinguishes hCG from the other glycoprotein hormones. It is composed of 145 amino acids linked by six disulfide bridges. The β-subunit contains two N-linked oligosaccharide side chains, attached to residues 13 and 30. It also has four O-linked oligosaccharide units, located in the unique proline- and serine-rich C-terminal extension (residues 122 to 145). A two-dimensional representation of the structure of hCG is illustrated in Fig. 1.

Serum and urine concentrations of biologically active hCG (nonnicked hCG) rise exponentially in the first trimester of pregnancy, doubling every 48 h, to a peak at about 10 weeks of gestation (weeks since last menstrual period). Concentrations decrease from the 10th to the 16th week of gestation, reaching approximately one-fifth of peak concentrations, and remain around this concentration until term (Fig. 2) *[1]*. The hormone is present in pregnancy serum and urine samples, along with a variety of dissociated or degraded hCG-related molecules that have little or no biological activity *[1–6]*.

Nicked hCG has a single cleavage in the β-subunit peptide, between residues 47 and 48, or less commonly between 43 and 44 or 44 and 45 (Fig. 1). Nicked hCG concentrations peak at the same time as nonnicked hCG concentrations, at around 10 weeks of pregnancy. Nicked hCG molecules account for approximately 9% of hCG molecules (mean proportion) in serum in the 2nd month of gestation. Proportions rise to 21% of hCG molecules (mean proportion) in the 9th month of normal pregnancy (Fig. 2). Similar proportions of nicked hCG are observed in urine samples *[1]*. Although these percentages are low, they can vary very greatly among individuals (Fig. 2). In a previous study of 176 first-trimester pregnancy serum samples, between 0% and 59% nicking was detected *[2]*.

Two forms of free α-subunit (free α) are present in serum and urine samples (Fig. 1). These include a regular free α, which is the same as that α-subunit of hCG, and a large free α. Large free α is hyperglycosylated, with larger, more-complex N-linked oligosaccharides *[7]*. The more-complex N-linked oligosaccharides prevent combination of large free α with β-subunit. As such, large free α is only produced by trophoblast cells as a free subunit,

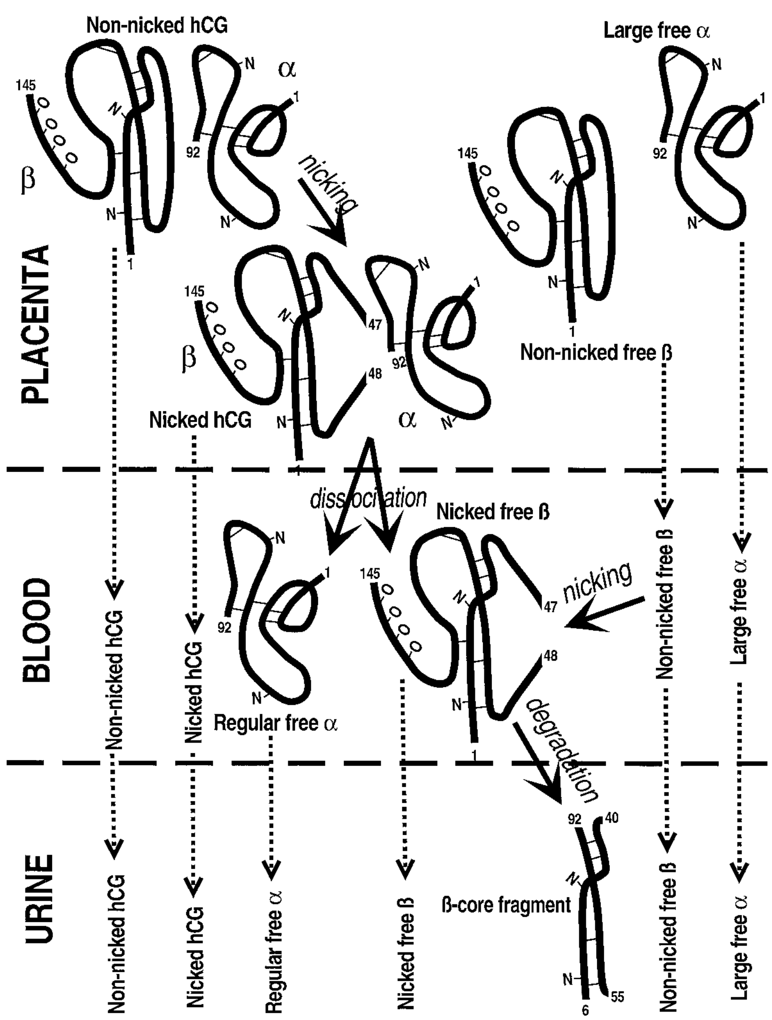

Fig. 1. Representation of the structures of hCG and related molecules in the placenta, blood, and urine.

Thick black lines represent the polypeptide chains. *Numbers* are the amino acid numbers in the chains. *Thin lines* represent disulfide linkages. Structures are folded according to the amino acid sequences of Morgan et al. *[40]*, and to match the disulfide linkages of Lapthorn et al. *[41]*. The nicking site is that determined by Kardana et al. *[5]*, and the structure of β-core fragment is that established by Birken et al. *[4]*. N- and O-linked oligosaccharides are indicated by the letters *N* and *O*, respectively. *Dotted arrows* indicate passage of molecules from the placenta into the circulation, and into urine. *Thick solid arrows* indicate nicking, dissociation, and degradation pathways.

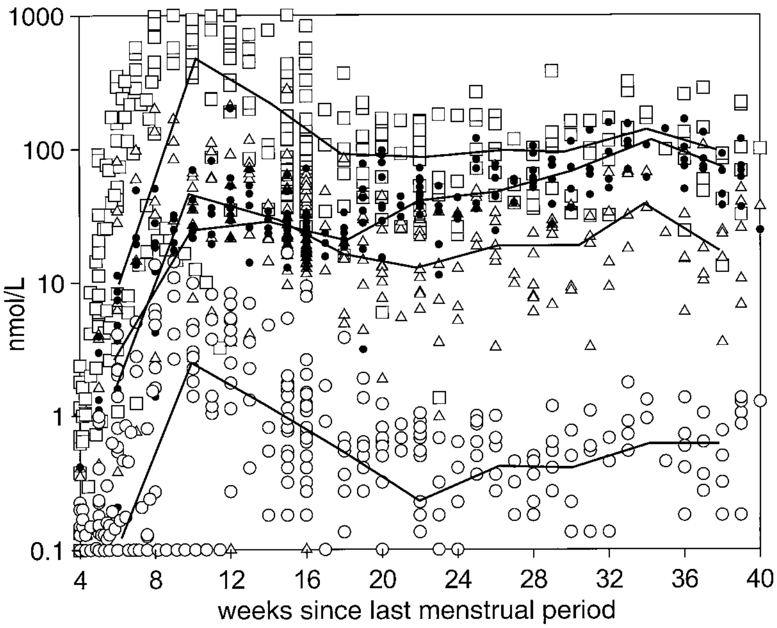

Fig. 2. Concentrations of hCG and its metabolites in pregnancy serum.

The concentration of nonnicked hCG (□), nicked hCG (△), free α (●), and free β (○) were determined by immunoassay in 300 serum samples, between 4 weeks and 40 weeks of gestation *[1–3]*. *Plotted lines* indicate median concentrations for 4-week periods. Starting from the top, in the final 4-week period, they represent nonnicked hCG, free α, nicked hCG, and free β, respectively. To illustrate the relative concentrations of analytes with different molecular and widely varying concentrations, the data are presented on a molar basis with a logarithmic scale.

and is not incorporated into hCG *[7]*. Electrophoresis studies indicate that the majority of free α molecules in pregnancy urine are large free α *[7]*. Currently, there are no immunoassays that discriminate large free α and regular free α concentrations. As such, we have to examine the two analytes together. The serum free α concentration is 5% of the hCG concentration (mean proportion) in the 2nd month of gestation. Proportions rise to 54% of the hCG concentration (mean proportion) in the 9th month of pregnancy (mol/mol) (Fig. 2). A somewhat higher proportion of free α-subunit may be observed in urine samples *[1]*. The proportion of free α molecules, like the nicked hCG molecules, varies widely (Fig. 2).

Nicked (nicked as hCG) and nonnicked free β are also present in serum and urine samples. Free β concentrations, like hCG concentrations, peak at around the 10th week of gestation. The total serum free β concentration is very low, 0.9% of the hCG concentration (mean proportion) in the 2nd month of gestation, declining to 0.5% (mean proportion) of the hCG concentration in the 9th month of pregnancy (Fig. 2) *[1]*. Higher proportions of free β (9% to 40% of hCG concentration, data not shown) may be observed in urine samples *[1]*.

β-core fragment is the terminal degradation product of hCG. Although it is the principal hCG β-subunit-related molecule in pregnancy urine samples, it is virtually undetectable in pregnancy serum (<0.3% of hCG concentration) *[4, 8]*. The β-core fragment comprises two peptides, β-subunit residues 6 to 40 and residues 55 to 92 held together by five disulfide linkages *[4]* (Fig. 1). β-core fragment (M_r = 9000) is approximately one-quarter of the size of hCG (M_r = 36 700) *[4]*. Urine β-core fragment concentrations follow the same general course as serum hCG concentrations, reaching a peak at around 10 weeks of gestation. β-core fragment concentrations start off lower than hCG concentrations. At 5 weeks of gestation they start to increase sharply, and at 6–7 weeks of gestation they equal hCG concentrations (mol/mol). β-core fragment concentrations exceed hCG concentrations thereafter (data not shown) *[1, 2]*. β-core fragment concentrations average 58% of urine hCG concentrations (mean proportion) in the 2nd month of pregnancy, rising to 305% of hCG concentrations in the final month of gestation (mean proportion) *[1, 2]*.

Nonnicked hCG, nonnicked free β, and large free α are secreted by isolated trophoblast cells in vivo *[3, 6–9]*. Nicked hCG, free β, and β-core fragment, however, are not secreted by trophoblast cells *[1, 6, 10]*. Cell culture and immunohistochemistry studies indicate that hCG is nicked after secretion by enzymes produced by macrophages associated with trophoblast cells *[1]*. Nicked hCG is unstable *[1, 12]*, rapidly breaking up into nicked free β and free α in serum. The virtual absence of β-core fragment in serum *[8]*, and its major presence in urine suggest that the β-core fragment is made in the kidney *[4, 8–10]*. Kinetic studies indicate that nicked free β is the substrate for β-core fragment synthesis in the kidney *[1, 11]*. A degradation pathway has been proposed for hCG: nonnicked hCG $\rightarrow$ nicked hCG $\rightarrow$ nicked free β $\rightarrow$ β-core fragment (Fig. 1) *[1, 11, 12]*.

Much greater and more variable proportions of nicked hCG, free β, and β-core fragment have been detected in Down syndrome pregnancies, preeclampsia, and trophoblast disease urine and serum samples *[13–16]*. Serum or urine containing entirely nicked hCG or free β and urine samples containing only β-core fragment have been found in certain trophoblast disease cases, testicular cancer or bladder cancer patients, and in normal pregnancy patients 3–10 days postpartum *[17–19]*. Nicking enzyme activity and the hCG degradation pathway are assumed to be more active in abnormal pregnancies, cancer and trophoblast disease, and in the days after clearance of hCG *[11, 12, 17]*.

hCG and Related Molecule Antibodies and Immunoassays

The old hCGβ RIA, with hCG β-subunit polyclonal antibody, generally measured all the different forms of the β-subunit of hCG (nicked and nonnicked hCG, free β, and β-core fragment) together, equally *[2]*. Times have changed, and automated and manual sandwich-type immunoassays with monoclonal antibodies and sophisticated spectrometric, lanthanide, or luminescence detection systems have replaced the old RIA. Depending on the mixture of monoclonal antibodies, these assays may measure differing mixtures of hCG-related molecules. Has hCG immunoassay technology advanced, or has its complicated itself by technology? It is one thing that we have heterogeneity in hCG, but it is yet another that we also have to deal with heterogeneity in what the hCG assay detects.

Multiple antibody binding sites have been identified on hCG and related molecules. As many as five separate antibody binding sites have been identified on nonnicked hCG, four separate sites on nicked hCG, two on free α, six on nonnicked free β, five on nicked free β, and as many as four separate sites on β-core fragment (Table 1). Most commercial hCG assays, whether for laboratory, office, or home use, include multiple antibodies raised to different sites on hCG and its free subunits (sandwich assays). Often, one monoclonal antibody is used to capture hCG through a specific site on the hormone. The immobilized or captured hCG is then detected by a separate antibody (monoclonal or polyclonal) raised against a distant site on the hormone. This antibody (tracer antibody) is labeled with a blue dye, with radioactivity, or with enzyme (for spectrometric or luminescence detection) to permit measurement of captured hCG. In some assays a second capture monoclonal antibody is used to capture free β. Free β is then detected by the same labeled antibody that detects hCG.

Manufacturers use a wide variety of different antibodies in their hCG immunoassay kits. As a result, not all hCG or hCGβ immunoassay kits measure the same thing. Some assays detect nonnicked hCG only (anti-hCG dimer capture antibody:anti-common β1 tracer antibody sandwich assays), some detect nonnicked hCG and free β (anti-hCG dimer plus anti-free β capture antibodies:anti-common β1 tracer antibody sandwich assays), others detect both nicked and nonnicked hCG (anti-common α capture antibody:anti-common β1 tracer antibody sandwich assays), and still others measure both forms of hCG and free β (anti-common α plus anti-free β capture antibodies:anti-common β1 tracer antibody sandwich assays, for instance). Still other assays detect all forms of hCG, free β-subunit, and β-core fragment (anti-common β1 competitive immunoassay, and certain anti-common β1 capture antibody:anti-common β2 tracer antibody sandwich assays). Table 2 lists some examples of quantitative serum hCG assays sold in the US, the antibody combination used, and what they are likely to detect (as indicated by instruction leaflets, by product management/technical support personnel, or in publications).

Our laboratory tested 15 serum samples from normal pregnancy and 15 serum samples from different patients with trophoblast disease, in seven different commercial hCG assays *[2]*. The assays included two competitive βhCG RIAs (Ortho-Clinical Diagnostics Amerlex M and Diagnostic Products HCG); two anti-common β1, anti-

Table 1. Commonly identified antibody binding sites (epitopes) on hCG, its free subunits, and degradation products.

		Reactivity						
Epitope	Descriptions	Nonnicked hCG	Nicked hCG	hCG-terminal	Nonnicked free β	Nicked free β	β-core fragment	Regular or large free α
Anti-hCG dimer	Site at subunit interface on nonnicked hCG	√						
Anti-common β1	Mutual site on hCG, free β, and β-core	√	√	√	√	√	√	
Anti-common β2	Separate mutual site on hCG and free β (β-core?)	√	√	√	√	√	±[a]	
Anti-β C-terminal	Mutual site on hCG and free β only	√	√		√	√		
Anti-common α	Mutual site on hCG and free α	√	√	√				
Anti-free β	Free subunit-specific site, hidden on hCG				√	√		
Anti-nonnicked free β	Free subunit-specific site, close to nicking site				√			
Anti-free β + β-core	Mutual site on free β and β-core fragment				√	√	√	
Anti-β-core fragment	β-core fragment-specific site, hidden on free β						√	
Anti-free α	Free subunit-specific site, hidden on LCG							√

[a] Some anti-common β antibodies also recognize β-core fragment.

common β2-type sandwich assays (Abbott 15/15 and Biomerica hCG); one anti-β C-terminal:anti-common β1-type sandwich assay (Organon NML); one anti-common α:anti-common β1-type sandwich assay (Hybritech Tandem-R); and one anti-hCG dimer plus anti-free β:anti-common β1-type sandwich assay (Serono MAIAclone) (Fig. 4). The assays were tested with a common pure hCG calibrator calibrated by amino acid analysis. The greatest assay-to-assay variation was 1.9-fold among the 15 pregnancy serum samples (Fig. 3, upper panel). This was found in sample 2. In this sample, the Diagnostic Products HCG assay result was 55 IU/L, and the Organon NML assay value was 102 IU/L. The CV was 9.9% for the 105 pregnancy determinations with seven assays (Fig. 3, upper panel). Larger assay-to-assay variation was found with trophoblast disease samples. Sample 1 was 1880 IU/L in the Ortho-Clinical Diagnostics Amerlex M and was 37 IU/L in the Organon NML assay (Fig. 3, lower panel). This was a 50-fold difference. Two- or more-fold difference in assays values were found in four of the 15 trophoblast disease samples. The CV was 17% for 105 determinations with seven assays.

Two types of assay gave particularly low or variable results with trophoblast disease serum samples. The Serono MAIAclone anti-hCG dimer + anti-free β:anti-common β1 sandwich assay detects nonnicked hCG molecules (the hormone) and free β. It gave consistently low results with trophoblast disease samples (Fig. 3, lower panel). With this assay, results for the 15 trophoblast disease samples were 75% (mean) of those found with the other six assays (mean). Similar low results have been found with other anti-hCG dimer-based assays *[2, 17]*. The Organon NML anti-β C-terminal:anti-common β1 sandwich assay, which detects molecules containing the C-terminal extension, gave sporadic results, in one case giving values 5.0% of the mean concentration. Similar results have been found with other anti-β C-terminal-based assays. Both of these types of assay gave good results with pregnancy serum, 82% to 96% and 86% to 130% of mean values. We infer that assays involving an anti-hCG dimer or an anti-β C-terminal-type antibody, while very appropriate for detecting pregnancy, may not be optimal for detecting hCG in patients with trophoblast disease.

Trophoblast disease samples typically contain unduly high proportions of nicked hCG and free β *[17]*. Some trophoblast disease hCG molecules lack the β-subunit C-terminal extension *[17]*. It is important when monitoring patients with trophoblast disease to use an assay that can detect all of these metabolites (Table 1). It is also important to tell the laboratory that very high hCG concentrations may be present (as in trophoblast disease and other pregnancy disorders). This way multiple dilutions can be used and the hook effect avoided (saturation of capture and label antibodies limiting sandwich formation, so that high hCG concentrations can give low results). Greater and more variable proportions of nicked hCG, free β, and β-core fragment have also been found in Down syndrome pregnancies, preeclampsia, and testicular and bladder cancer patients *[13–16]*. Similar care must be taken in selecting a hCG assay (or hCG testing center) for samples from these disorders.

Unduly high proportions of nicked hCG, free β, and β-core fragment have been noted in serum and urine samples during clearing of hormone, 3 to 10 days postpartum, or after termination of pregnancy. Similar care is required in choosing an assay to measure these molecules in monitoring completeness of evacuation *[17]*. Fig. 4 shows concentrations of nonnicked hCG and of both forms of hCG after evacuation of a hydatidiform mole (trophoblast disease). Concentrations determined by an assay measuring nonnicked hCG reached baseline concentrations (3 IU/L) rapidly (day 25), whereas those determined with an assay measuring both forms of hCG were still increased (and may indicate the persistence of trophoblast disease) and reached baseline concentrations considerably later. Similar results have now been observed in our laboratory after the evacuation of 13 of 17 hydatidiform moles, after chemotherapy for choriocarcinoma, and after parturition in four term pregnancies *[17*, L. Cole, unpublished results*]*. A shift from nonnicked to nicked hCG is inferred to occur in later weeks after therapy of trophoblast disease or after normal pregnancy parturition. Whether the residual nicked hCG represents the presence of trophoblast cells, necrotic trophoblast cells, or the slow degradation and clearance of hCG remains to be determined.

β-core fragment is the principal form of hCG β-subunit in pregnancy urine samples. It is detected by the anti-common β1 RIA or enzyme immunoassay, and by certain anti-common β2:anti-common β1-type assays (check with manufacturer). Large variation is found in individual results when including or not including β-core fragment in urine hCG determinations. The addition of β-core fragment to pregnancy hCG concentrations raises concentrations from as little as 1.02-fold to as much as 26.5-fold *[2]*. In the second month of pregnancy, when most pregnancy tests are performed, the concentration of hCG plus β-core fragment is approximately twice that of hCG alone *[2]*. It is important to be aware of this large difference, and the incompatibility of both quantitative and qualitative results from tests including and excluding β-core fragment. Monitoring pregnancy urine with hCG-only tests, and with those including free β and β-core fragment are equally valid, but yield very incomparable results. As a general rule, both serum and urine hCG immunoassay results are assay specific. Results from one particular assay, one hospital, or a single testing center should be trusted, and not compared or used in conjunction with those from another immunoassay or site.

Persistent Low Concentrations of hCG

Persistent low hCG immunoassay results have been reported postpartum or postmenopause; they have been

Table 2. Examples of quantitative immunoassay kits detecting hCG, its free subunits, and degradations products.

Manufacturer	Kit	Antibody types[a] Capture antibody:labeled antibody[b]	Specificity[a]
hCG and hCGβ assays			
Abbott Labs.	IMX hCG	Anti-common α:anti-β C-terminal	Nicked + nonnicked hCG
Abbott Labs.	IMX βhCG	Anti-β C-terminal:anti-common β1	Nicked + nonnicked hCG + free β
Abbott Labs.	AxSYM βhCG	Anti-β C-terminal:anti-common β1	Nicked + nonnicked hCG + free β
Ortho Diagnostics	Amerlex-M	Competitive, anti-common β1	Nicked + nonnicked hCG + free β + β-core
Baxter Diagnostics	Stratus hCG	Anti-hCG dimer:anti-common β1	Nonnicked hCG only
Baxter Diagnostics	Stratus βhCG	Anti-common β2:anti-common β1	Nicked + nonnicked hCG + free β (+ β-core?)
Bayer Diagnostics	Technicon Immuno-1	Anti-hCG dimer + anti-free β:anti-common β1	Nonnicked hCG + free β
Binax	hCG	Anti-common α:anti-common β1	Nicked + nonnicked hCG
Binax	βhCG RIA	Competitive, anti-common β1	Nicked + nonnicked hCG + free β + β-core
Bioclone Australia	HCG IRMA	Anti-hCG dimer:anti-common β1	Nonnicked hCG only
Bioclone Australia	βHCG ELISA	Anti-hCG dimer + anti-free β:anti-common β1	Nonnicked hCG + free β
Biomerica	hCGβ	Anti-common β2:anti-common β1	Nicked + nonnicked hCG + free β (+ β-core?)
BioMerieux Vitek	hCG	Anti-hCG dimer:anti-common β1	Nonnicked hCG only
Bio-Rad Labs.	Cotube hCG IRMA	Anti-common α:anti-common β1	Nicked + nonnicked hCG
BiosPacific	hCG Pregnancy	Anti-common α:anti-common β1	Nicked + nonnicked hCG
Boehringer Mannheim	ES300 Enzymum hCG	Anti-common β2:anti-common β1	Nicked + nonnicked hCG + free β (+ β-core?)
Chiron Diagnostics	Magic Light	Anti-common α + anti-free β:anti-common β1	Nicked + nonnicked hCG + free β
Chiron Diagnostics	ACS180	Anti-common α + anti-free β:anti-common β1	Nicked + nonnicked hCG + free β
Diagnostic Products	hCG IRMA	Anti-hCG dimer:anti-common β1	Nonnicked hCG only
Diagnostic Products	βhCG RIA	Competitive, anti-common β1	Nicked + nonnicked hCG + free β + β-core
Diagnostic Products	Immunolite hCG	Anti-common β2:anti-common β1	Nicked + nonnicked hCG + free β
Dupont	ACA hCG	Anti-common α:anti-common β1	Nicked + nonnicked hCG
Guildhay	hCG ELISA	Anti-common α:anti-common β1	Nicked + nonnicked hCG
Hybritech	Tandem-R/-E hCG	Anti-common α:anti-common β1	Nicked + nonnicked hCG
Hybritech	Tandem-R total β	Anti-common α + anti-free β:anti-common β1	Nicked + nonnicked hCG + free β
ICN Biomedicals	hCGβ RIA	Competitive, anti-common β1	Nicked + nonnicked hCG + free β + β-core
Immunonuclear	Gammadab bHCG	Competitive, anti-common β1	Nicked + nonnicked hCG + free β + β-core
Medix Biotech	hCG (intact) EISA	Anti-hCG dimer:anti-common α	Nonnicked hCG only
Medix Biotech	hCG (intact) EISA	Anti-common β2:anti-common β1	Nicked + nonnicked hCG + free β (+ β-core?)
Nichols Diagnostics	Isotopic	Anti-hCG dimer + anti-free β:anti-common β1	Nonnicked hCG + free β
Nichols Diagnostics	Chemiluminescent hCG	Anti-common β2:anti-common β1	Nicked + nonnicked hCG + free β (+ β-core?)
Organon Teknika	NML	Anti-β C-terminal:anti-common β1	Nicked + nonnicked hCG + free β
PB Diagnostics	Opus hCG	Anti-common α:anti-common β1	Nicked + nonnicked hCG
Sanofi Diagnostic	Access system βhCG	Anti-common β2:anti-common β1	Nicked + nonnicked hCG + free β (+ β-core?)
SeaLite Sciences	AquaLite hCG	Anti-hCG dimer:anti-common β1	Nonnicked hCG only
Serono Diagnostics	MAIAclone	Anti-hCG dimer + anti-free β:anti-common β1	Nonnicked hCG + free β
Sunita Chemicals	hCG	Anti-hCG dimer:anti-common β1	Nonnicked hCG only
Syntron Bioresearch	MicroCheck	Anti-hCG dimer:anti-common β1	Nonnicked hCG only
Tosoh Medics	hCG	Anti-hCG dimer:anti-common β1	Nonnicked hCG only

Table 2. Continued.

Manufacturer	Kit	Antibody types[a] Capture antibody:labeled antibody[b]	Specificity[a]
hCG and hCGβ assays			
Tosoh Medics	βhCG	Anti-common β2:anti-common β1	Nicked + nonnicked hCG + free β (+ β-core?)
V-Tech	Target hCG	Anti-common α:anti-common β1	Nicked + nonnicked hCG
Wallac	Delfia hCG	Anti-common α:anti-common β1	Nicked + nonnicked hCG
Free subunit and fragment assays			
Bayer Diagnostics	Technicon Immuno-1	Anti-β core fragment:anti-common β1	β-core fragment only
Bioclone Australia	Free β-subunit	Anti-free β:anti-common β1	Nicked and nonnicked free β
Bioclone Australia	Free α-subunit	Anti-free α:anti-common α	Free α
Chiron Diagnostics	UGP β-core fragment	Anti-β core fragment:anti-common β1	β-Core fragment only
Cis	ELSA-F free β-subunit	Anti-free β:anti-common β1	Nicked and nonnicked free β
Diagnostic Products	Free β-subunit	Anti-free β:anti-common β1	Nicked and nonnicked free β
Vitros Immunodiagnostic	Free β-hCG	Anti-free β:anti-common β1	Nicked and nonnicked free β
Metachem Diagnostics	Free α-subunit	Anti-free α:anti-common α	Free α
Toagosei Co.	UGF β-core fragment	Anti-β core fragment:anti-common β1	β-Core fragment only
Waco	β-core	Anti-free β + β core fragment:anti-common β1	β-Core fragment + free β equally
Wallac	Delfia free β-subunit	Anti-free β:anti-common β1	Nicked and nonnicked free β
Wallac	Delfia β-core fragment	Anti-β core fragment:anti-common β1	β-Core fragment only

[a] As indicated in instruction booklets, by telephone technical support services, or in published reports.
[b] In either order.

detected in men and in nonpregnant women (concentrations 3 to 100 IU/L, or 0.3 to 10 μg/L). Persistent low concentrations can come from a variety of sources. The gonadotroph cells of the pituitary secrete low amounts of hCG in men and women. Normal pituitary hCG can account for as much as 3 IU/L (0.3 μg/L) of serum hCG *[20–22]*. Rarely, normal pituitary or pituitary adenoma can be the source of unduly high hCG concentrations (up to 100 IU/L). Pituitary hCG production may in some cases be quenched with sex steroids or controlled by gonadoliberin (GnRH) *[20]*. Treatment with progesterone or GnRH analogs could be used to identify pituitary hCG production.

Trophoblast disease must be considered a possible source for low persistent concentrations of hCG in postpartum women *[17]*. In all nonpregnant individuals, testicular cancer, ovarian cancer, bladder cancer, or other malignancy must be ruled out as a source for low concentrations of serum hCG or free β, or urine β-core fragment *[23, 24]*. One explanation for persistent low concentrations of hCG is phantom hCG. Phantom hCG immunoreactivity can be produced by some trypsin-like molecules, cholera toxin, transforming growth factor-β, or by hCG immunoreactive molecules produced by certain bacteria *[25, 26]*. Generally, phantom hCG does not give a parallel dose–response in hCG immunoassays. Testing multiple serum dilutions in the immunoassay may identify this phenomenon.

Free Subunit and β-Core Fragment Immunoassays

During the past 5 years, new applications have emerged for specifically measuring hCG free subunits and their metabolites. Commercial immunoassays have now been introduced for measuring free β, free α, and β-core fragment. Table 2 lists some of these new commercial assays and their specificities.

Over 10 years ago, free β measurements were shown to be useful in the diagnosis and management of trophoblast disease *[18, 19]*. More recently, raised free β concentrations have been use to screen pregnancies for Down syndrome fetuses *[12, 27]*. Free β has also been indicated as a superior tumor marker for testicular cancer *[23]*, and possibly other malignancies *[28]*. Serum nicked free β has been suggested as an alternative screening test for Down syndrome *[29]*.

β-core fragment, the urine degradation product of nicked free β, is being developed as a high-efficiency screening test for Down syndrome pregnancies *[14, 15, 30]*. As a single test, β-core fragment may be more effective than free β and the triple screen test, a complex of three tests, for Down syndrome screening *[14, 15, 30]*. β-core fragment immunoassay kits have been approved in certain countries for use in detecting β-core fragment as a tumor marker and for following the therapy of ovarian, bladder, or cervical malignancies *[23, 31–33]*.

Two companies sell immunoassay kits for specifically measuring free α (Table 2). Few applications have been described, however, for free α measurement. It has been

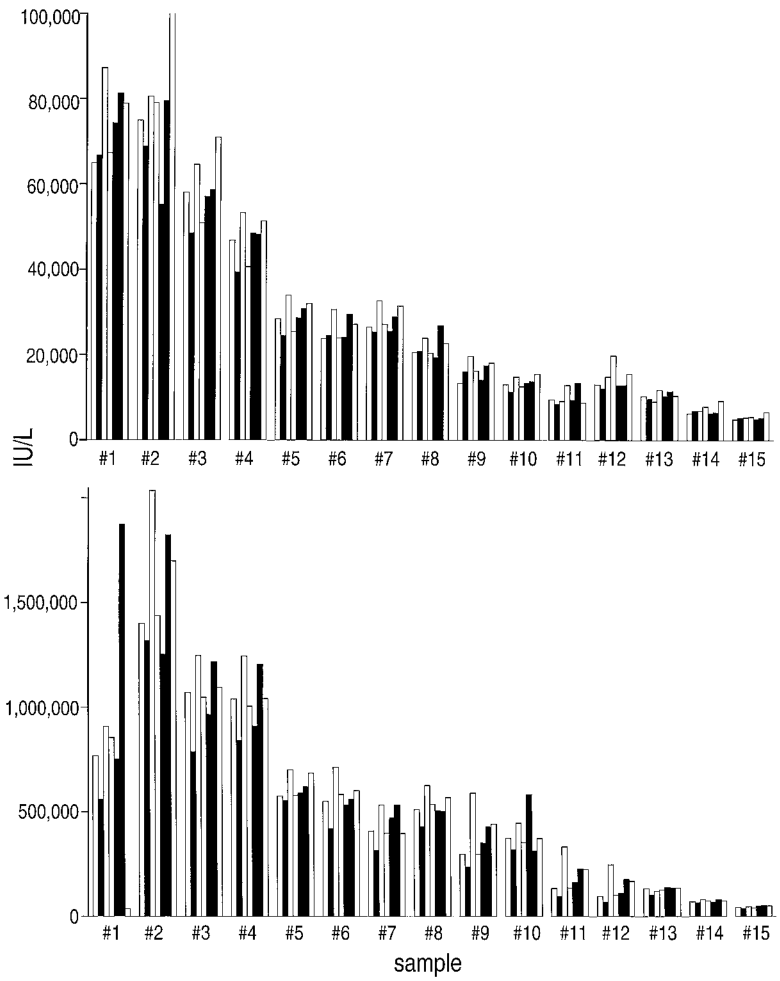

Fig. 3. Interassay discordance in hCG determinations.

Fifteen serum samples from first- or second-trimester pregnancy *(upper panel)* and 15 from trophoblast disease *(lower panel)* were each tested with seven different commercial hCG immunoassays. From left to right the bars are Hybritech Tandem-R kit (*open bar*), Serono MAIAclone kit (*solid bar*), Abbott hCGβ 15/15 and Bio-merica hCGβ kits (*open bars*), Diagnostic Products HCG and Ortho-Clinical Diagnostics Amerlex M kits (*solid bars*), and Organon NML kit (*open bar*). Data are presented as IU/L.

suggested as a marker of Down syndrome pregnancies. The use in this application, however, may be very limited *[34]*. hCG free α is immunologically indistinguishable from lutropin, follitropin, and thyrotropin free α. This limits the use of hCG free α measurements, and its use a tumor marker or as a simple pregnancy test.

Stability of hCG, Free β, and β-Core Fragment

Nonnicked hCG is a very stable molecule if preserved or kept sterile in blood or serum. In 1982, a dissociation half-time of approximately 700 h *[22]* was suggested for hCG at 37 °C *[35]*. More recently, a dissociation half-time of 8 weeks (1300 h) was shown for nonnicked hCG at the same temperature *[36]*. As shown in Fig. 5, pure nonnicked hCG dissociated at a rate of 14% ± 1.4% per week in antibiotic-preserved serum at 37 °C. It dissociated at a much faster rate, however, 34% ± 5.6% per week, in similarly preserved urine.

Low temperatures have very little effect on nonnicked hCG concentrations. After 4 weeks at 21 °C or 4 °C, very little change was found in sterile/preserved serum hCG concentrations, 94% ± 3.1% and 94% ± 8.3%, respectively *[12]*. The bulk of the decrease may be attributed to hCG nicking, and more rapid dissociation of nicked hCG to free subunit *[12]*. The hCG calibrator in many commercial immunoassay kits has a significant nicked hCG component. A proportion of these nicked molecules, and those generated by nicking in the refrigerator, will dissociate to free subunits in the refrigerator *[12]*. If your assay detects both forms of hCG and free β, the results will not be affected by this nicking dissociation process.

Free β is an extremely minor component of normal pregnancy serum hCG, <1% of the hCG concentration (Fig. 2). If your objective is to measure normal pregnancy hormone, there is no reason to use an assay detecting free β, except to accommodate the dissociation of the nicked or nonnicked hCG to free subunits over a 2-week or longer period in the refrigerator. Because free β concentrations are so low in pregnancy serum, they can be flooded by β-subunit from the dissociation of nicked and nonnicked hCG *[12, 36, 37]*. As shown in Fig. 5, free β in normal first-trimester pregnancy serum and urine samples may be amplified 20- to 30-fold during 1 week of storage or a similar shipping period at body-like temperatures (in presence of antibiotics) *[36]*. β-core fragment is a more stable molecule. No measurable change was observed in

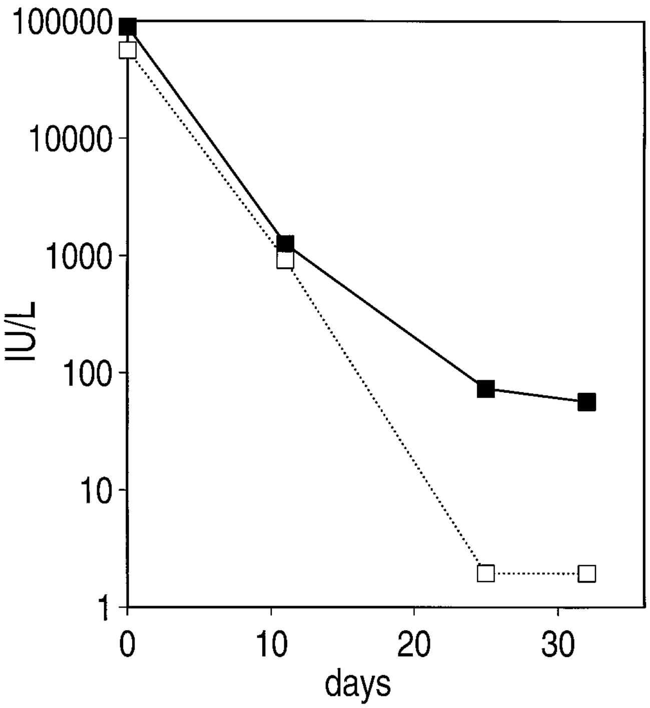

Fig. 4. Clearance of nicked and nonnicked hCG after evacuation of a complete hydatidiform mole.

Serum hCG concentrations were measured by two immunoassays, one measuring nonnicked hCG only (□) and an assay measuring both forms of hCG (■), in the days after therapy for hydatidiform mole.

normal first-trimester pregnancy urine after 7 days at 37 °C (in presence of antibiotics) *[36]*.

hCG and Related Molecule Standards

Currently, two international standards are used for calibrating hCG assays, the First International Reference Preparation for immunoassay and the Third International Standard for hCG (established 1986) *[38]*. Both standards are made from the same large preparation of completely pure hCG (preparation CR119, originally prepared by Canfield and Birken at Columbia University, New York, and donated to WHO). Sequence analysis shows that these pure hCG preparations contain 9% nicked hCG and 91% nonnicked hCG *[39]*. WHO distributes these immunoassay standards by weight, and calibrates them in IU, where 1 μg of pure hCG = 9.3 IU *[38]*. Manufacturers receive small quantities of one of the two WHO International Standards, and use them to calibrate large quantities of crude urinary hCG (organic extract, partially purified hCG, or in a few cases 95% pure hCG). These crude urinary calibrators may contain significant proportions of nicked hCG, free β, or β-core fragment. Thus the calibrator is also assay specific. One kit calibrator may be 100 IU/L in one assay (that measures nonnicked hCG only), but would be effectively 300 IU/L if used with a different kit (that measures both forms of hCG plus free β and β-core fragment). New International Standards are on the horizon, involving nonnicked recombinant DNA technology hCG.

International Standards have also been prepared for free α and free β. These are also weighed out, but with the formula 1 μg = 1 IU *[38]*. They are somewhat incompatible with hCG standards, since 1 IU of free β represent 0.045 nmol of free β, and 1 IU of hCG represents 0.0029 nmol of hCG. As such, 1 IU of free β contains 15.5-fold more β-subunit than 1 IU of hCG. No International Standard has been established as yet for β-core fragment.

Summary and Recommendations

Multiple hCG-related molecules are present in pregnancy serum and urine samples. These may differ widely in peptide or carbohydrate structure, and in their recognition by different hCG immunoassays. Care is needed in choosing an hCG immunoassay for a hospital, clinic, or commercial laboratory. Consideration must be made not just of assay speed, proprietary machine, and assay cost, but of exactly what the assay is detecting. Some assays detect only nonnicked hCG, the biologically active hormone. Others detect nonnicked hCG and free β. This is an odd mixture of molecules since it excludes the significant intermediate, nicked hCG. Still other assays detect both nicked and nonnicked hCG or all forms of hCG, and other assays detect all forms of hCG and free β. Further assays detect all forms of hCG, free β, and β-core fragment. Some assays include an antibody to β-subunit C-terminal extension, and do not recognize the rarer molecules missing this extension. All these types of assay are excellent for detection of normal pregnancy. Abnormal pregnancies (trophoblast disease, Down syndrome pregnancies, preeclampsia, and testicular and bladder cancers) may produce a much larger proportion of degraded or dissociated hCG molecules. In some cases, only nicked hCG or only free β-subunit is present in the circulation. Detection of these molecules (nicked hCG, free β, nicked free β, and molecules missing the β-subunit C-terminal segment) may be much more important in abnormal pregnancy hCG applications. Only assays that detect these degraded molecules may be recommended for abnormal pregnancy applications.

A new labeling system is needed for hCG assays to clarify what they are detecting. Currently, assays are labeled "intact hCG," "total hCG," or "hCGβ." This labeling is both confusing and inadequate. Is nicked hCG "intact hCG?" Is hCG missing the β-subunit C-terminal segment "intact hCG?" Is nonnicked hCG and free β really "total hCG?" hCG immunoassays could be more clearly labeled "nonnicked hCG only (or hormone only)," "nonnicked hCG plus free β (or hormone plus free β)," "nicked and nonnicked hCG (or whole hCG)," "nicked and nonnicked hCG plus free β (or whole hCG plus free β)," etc. Using such a system, physicians could better compare immunoassay results from different laboratories, and more correctly order the appropriate test for a problem pregnancy.

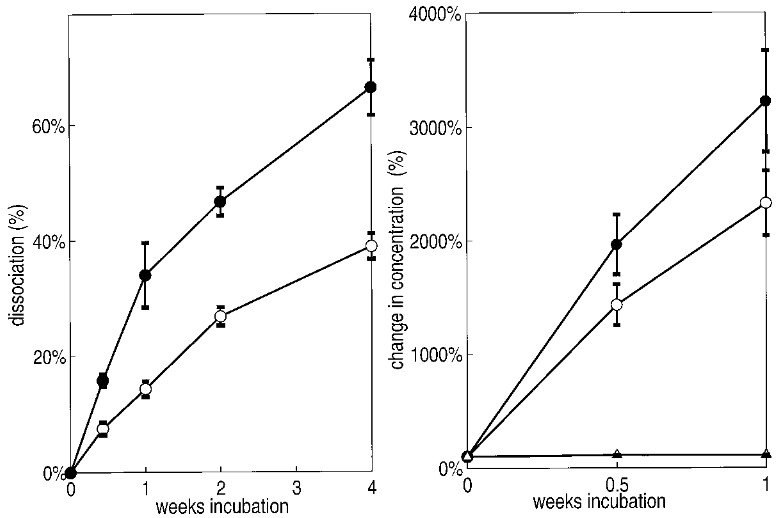

Fig. 5. Stability of hCG and its metabolites in serum and urine samples *[35]*.

The *left panel* shows the mean dissociation (±SE) at 37 °C of three pure preparations of nonnicked hCG, each added to pooled serum (○) or urine (●) freshly collected from six nonpregnant individuals (+ antibiotics). Percent dissociation was measured by the liberation of free β according to the equation free β (μg/L) × molecular mass factor (1.65)/hCG at start (μg/L). The *right panel* shows changes in free β concentration in six normal first-trimester pregnancy serum samples (○) and urine (●) samples (% ±SE), and in urine β-core fragment concentration (△) after incubation at 37 °C (+ antibiotics) *[35]*.

New immunoassays are now available, detecting hCG free subunits and β-core fragment. Applications are emerging for measuring these molecules, particularly in the detection and management of abnormal pregnancies, and as tumor markers. Care is again needed in choosing an assay that measures the molecule in question. Immunoassays should be labeled appropriately. Assays that measure free β plus β-core, for instance, should be labeled as such (the Waco β-core kit, for instance, measures free β and β-core equally). Certain manufacturers have given β-core fragment different names, urinary gonadotropin peptide and urinary gonadotropin fragment. I must admit I had some part in deriving these odd names. When I see papers from different groups calling the same molecule completely different things, I realize that the different names are yet another source of confusion.

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