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# HYPERGLYCOSYLATED hCG, A POTENTIAL ALTERNATIVE TO hCG IN DOWN SYNDROME SCREENING

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## SUMMARY

Hyperglycosylated hCG (H-hCG) is a minor variant of hCG with abnormal oligosaccharide side chains. It is the principal gonadotropin detected in the serum and urine of patients with gestational choriocarcinoma. A monoclonal antibody was produced against this antigen and an immunoassay developed. Levels of hCG and H-hCG were determined in 142 urine samples from normal pregnancies from 10 to 21 weeks of gestation. Levels were normalized to urine creatinine concentration, and were each plotted against gestational age. Bi-weekly median values were calculated, the best-fitting regression lines were determined, and multiples of the normal median (MoM) were computed.

10 Down syndrome pregnancy samples were tested from 11 to 21 weeks of gestation. The median hCG and H-hCG levels in the Down syndrome cases were 1·9 MoM and 5·7 MoM of unaffected cases, respectively. Four of 10 hCG measurements and 9 of 10 H-hCG determinations exceeded the 95th centile of unaffected cases.

H-hCG identified 90 per cent of Down syndrome cases with a 5 per cent false-positive rate. This is more than twice the number of cases detected by an hCG assay. H-hCG may be an effective replacement for hCG in antenatal Down syndrome screening. This is a preliminary report consisting of only 152 samples. Further studies are needed now to verify the Down syndrome screening utility of this potentially valuable new marker. © 1998 John Wiley & Sons, Ltd.

## INTRODUCTION

In the 1980s three serum tests were identified as markers of Down syndrome fetuses in the second trimester of pregnancy. These were hCG (Bogart *et al.*, 1987), and two less-discriminating tests, AFP (Merkatz *et al.*, 1984) and unconjugated oestriol (Wald *et al.*, 1988a). The three tests were combined to obtain the maximal Down syndrome screening performance (Wald *et al.*, 1988b; Canick, 1990). These three tests (triple test) are now widely used for screening for Down syndrome between 15 and 20 weeks of gestation.

The triple test is far from perfect. Its use is limited to second-trimester pregnancies (Wald *et al.*, 1998b; Canick, 1990). It has a low detection rate, failing to identify 35 to 40 per cent of Down syndrome cases (Canick, 1990), and a relatively high false-positive rate (5 per cent). Cost has also become a limitation of the triple test (Auxter, 1997; Zoler, 1997), with significant licence fees levied on laboratories running hCG screening tests. New tests are needed to replace hCG and the triple test. Such tests might detect Down syndrome in the first trimester, or both the first and second trimesters of pregnancy, and offer a significant improvement in

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the false-positive or detection rate over current Down syndrome screening procedures.

Recently, we purified hCG, free $\beta$-subunit and $\beta$-core fragment from large volumes of urine from individuals with normal pregnancy, with Down syndrome pregnancy and from women with gestational choriocarcinoma. We examined the polypeptide sequences of these preparations (Elliott *et al.*, 1997; Kardana *et al.*, 1991; Cole *et al.*, 1997a). No difference was found in the N-terminal peptide sequences (amino acid residues 1–10) or in the amino acid analyses of hCG, free $\beta$-subunit or $\beta$-core fragment, whether from normal pregnancy, Down syndrome pregnancy or gestational choriocarcinoma.

We investigated the compositions of the N-linked and O-linked oligosaccharide side chains on the purified hCG preparations. The six normal pregnancy hCG preparations had primarily mono- and bi-antennary N-linked oligosaccharides, and trisaccharide and tetrasaccharide-type O-linked sugar units. The five choriocarcinoma hCG preparations, however, had primarily larger more-complex oligosaccharides with additional sialyl N-acetyllactosamine antennae (hyperglycosylated oligosaccharides) (Elliott *et al.*, 1997).

We used lectin affinity chromatography to investigate the occurrence of hyperglycosylated oligosaccharides on hCG free $\beta$-subunit in Down syndrome pregnancies. Hyperglycosylated oligosaccharides were found on a small proportion of molecules in 18 of 109 (17 per cent) normal pregnancy and 9 of 15 (60 per cent) Down syndrome urine samples (Cole *et al.*, 1997a). We investigated the composition of N-linked oligosaccharides on purified $\beta$-core fragment preparations. Hyperglycosylated oligosaccharides (heptasaccharides) were found on 2 of 2 samples from gestational choriocarcinoma, 2 of 2 samples from Down syndrome pregnancies and 0 of 2 samples from normal pregnancy samples (Cole *et al.*, 1997a). We infer that hyperglycosylated oligosaccharides are a property of hCG (hyperglycosylated hCG, H-hCG), and hCG free subunits and fragments, produced in women with gestational choriocarcinoma. They may also be present in raised portions of molecules from pregnancies with Down syndrome fetuses.

We purified H-hCG from the urine of a patient with advanced choriocarcinoma (hCG preparation C5) (Kardana *et al.*, 1991). In collaboration with R. Canfield, S. Birken, A. Kichevsky and J. O'Connor of Columbia University, New York, monoclonal antibodies were prepared against hCG preparation C5. A clone was identified detecting hyperglycosylated molecules, and a specific immunoassay was established to measure H-hCG. We report here the use of this assay to detect pregnancies with Down syndrome fetuses. H-hCG levels are compared with hCG levels, and the portion of hyperglycosylated molecules determined.

## MATERIALS AND METHODS

Urine samples were collected from 1232 women undergoing fetal karyotype testing because of advanced maternal age. Collection and consent procedures have been described previously (Cole *et al.*, 1997b; Isozaki *et al.*, 1997). All samples were refrigerated upon collection, aliquoted and frozen at $-20$°C in the laboratory. Personal information, assay results, gestational ages (based on ultrasound) and karyotype were recorded in a computer spreadsheet (Cole *et al.*, 1997b; Isozaki *et al.*, 1997). For this study we tested 152 urine samples. These were the first 142 control samples, and the first 10 Down syndrome cases in our spreadsheet.

H-hCG levels were determined in a two-step sandwich assay. This used the C5 hCG-directed antibody as the capture antibody, and monoclonal antibody 4001 (anti-hCG$\beta$), peroxidase-labelled (Genzyme, San Carlos, CA, U.S.A.), as tracer antibody, and our previously described methods (Cole *et al.*, 1993). Pure H-hCG (choriocarcinoma hCG batch C7, calibrated by amino acid analysis) was used to standardize the assay. hCG (intact hCG) levels were determined by a similar immunoassay, using parallel methods. This assay used monoclonal antibody 2119 (anti-hCG$\alpha$) as capture antibody, antibody 4001 (anti-hCG$\beta$), peroxidase-labelled, as tracer, and NIH CR127 hCG as standard. hCG and H-hCG levels (ng/ml) were normalized to urine creatinine concentration (ng/mg creatinine), as described previously (Isozaki *et al.*, 1997).

Results were analysed using standard statistical methods (Royston and Thompson, 1992). hCG and H-hCG levels were each plotted against gestational age. Bi-weekly median values were calculated, the best-fitting regression lines were determined and MoM values were computed. To assess screening performance, medians and mean values were calculated, and observed detection rates and false-positive rates were recorded. The proportion of hyperglycosylated molecules was

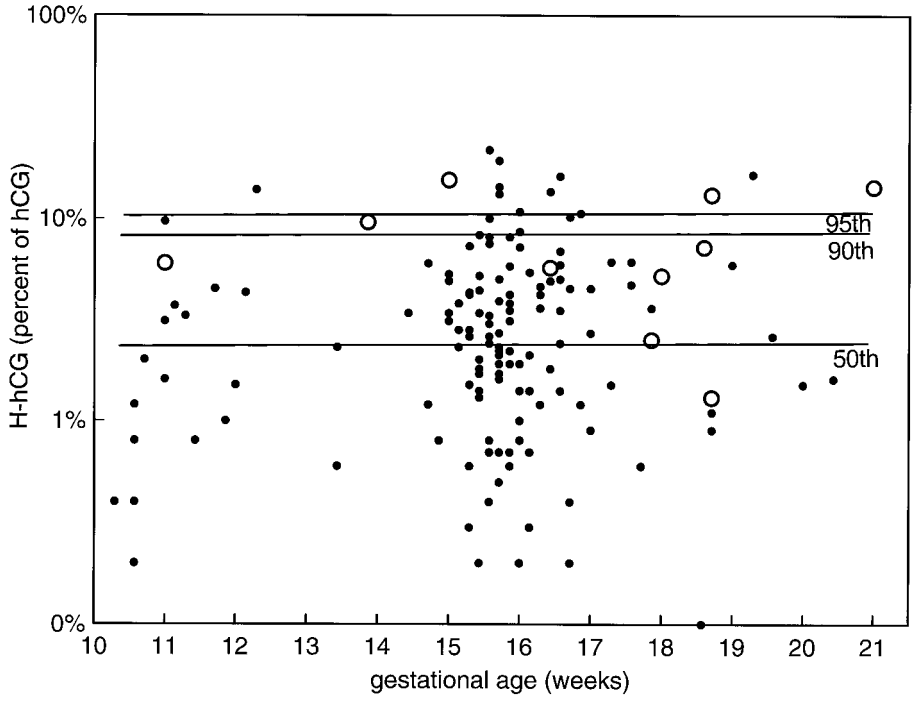


Fig. 1—hCG levels (ng/mg creatinine) in 142 normal (●) and 10 Down syndrome (○) pregnancy urine samples

estimated (H-hCG (ng/ml)÷hCG (ng/ml)), and compared in control and aneuploid cases.

## RESULTS

Urine hCG levels were determined and normalized to creatinine concentration (ng hCG/mg creatinine). Results were plotted against gestational age and bi-weekly medians were determined (Fig. 1). The medians best fit a logarithmic line. The equation for the line was median = $5695 \times 0{\cdot}886^{ga}$ (ga = gestational age). The median MoM was 1·00. There was no significant affect of fetal sex on this median. Ten Down syndrome pregnancies were tested from the same gestational period (Table I). The median MoM for the Down syndrome cases was 1·9, indicating a 1·9-fold increase in hCG levels. Urines from 4 of 10 Down syndrome cases exceeded the 90th centile and 95th centile of the unaffected cases. 40 per cent detection was therefore observed at a 5 per cent false-positive rate.

Levels of H-hCG were determined in the same urine samples. Values for normal karyotype cases were plotted against gestational age and bi-weekly medians were determined (Fig. 2). The medians all best fit a logarithmic line. The equation for the line was median = $269 \times 0{\cdot}849^{ga}$. The median MoM was 1·01. There was no significant affect of fetal sex on this median. The median MoM for the Down syndrome cases was 5·7, indicating a 5·7-fold increase in levels. Urines from 10 of 10 Down syndrome cases exceeded the 90th centile, and 9 of 10 exceeded the 95th centile of the unaffected cases (Table I). 90 per cent detection was therefore observed at a 5 per cent false-positive rate.

To assess H-hCG independently, we calculated extent of hyperglycosylation of hCG (percentage of hCG molecules recognized by the H-hCG assay). Results for normal cases were plotted against gestational age and bi-weekly medians were determined (Fig. 3(a)). A small incline was observed in the percentage of H-hCG molecules with advancing gestational age. The H-hCG assay recognized 1·6 per cent (median) of hCG molecules in normal first-trimester urine samples, and 2·7 per cent of molecules in second-trimester samples ($P = 0{\cdot}90$, not significant). The medians all best fit a logarithmic line. The equation for the line was median = $0{\cdot}0164 \times 1{\cdot}02^{ga}$. The median MoM was

 

Table I—Screening performance of hCG and H-hCG immunoassays, and percentage hyperglycosylated hCG measurements

| | | H-hCG | | | hCG | | | Extent of hyperglycosylation* | | |
|---|---|---|---|---|---|---|---|---|---|---|
| GA | Karyotype | ng/mg creatinine | MoM | Centile | ng/mg creatinine | MoM | Centile | % | MoM | Centile |
| 16 weeks 3 days | 47,XX,+21 | 65·9 | 3·6 | 94·5 | 1151 | 1·5 | 75·4 | 5·7 | 2·5 | 5·2 |
| 18 weeks 0 days | 47,XY,+21 | 58·3 | 4·1 | 97·3 | 1122 | 1·7 | 81·0 | 5·2 | 2·2 | 78·9 |
| 17 weeks 6 days | 47,XY,+21 | 61·1 | 4·2 | 97·4 | 2456 | 3·8 | 98·4 | 2·5 | 1·1 | 50·7 |
| 18 weeks 5 days | 47,XX,+21 | 66·7 | 5·3 | 98·6 | 5319 | 9·0 | >99 | 1·3 | 0·5 | 24·8 |
| 11 weeks 0 days | 47,XX,+21 | 245 | 5·5 | 98·7 | 4093 | 2·7 | 95·4 | 6·0 | 2·9 | 86·6 |
| 18 weeks 4 days | 47,XY,+21 | 75·0 | 5·9 | 99·0 | 1044 | 1·7 | 81·4 | 7·2 | 3·0 | 87·3 |
| 21 weeks 0 days | 47,XY,+21 | 63·0 | 7·3 | >99 | 442 | 0·99 | 49·8 | 14 | 5·7 | 96·2 |
| 15 weeks 0 days | 47,XX,+21 | 194 | 8·4 | >99 | 1256 | 1·4 | 68·9 | 16 | 7·0 | 98·8 |
| 18 weeks 5 days | 47,XX,+21 | 167 | 13·3 | >99 | 1273 | 2·2 | 98·8 | 13 | 5·5 | 95·8 |
| 13 weeks 6 days | 47,XX,+21 | 927 | 33·3 | >99 | 9639 | 9·1 | >99 | 9·6 | 4·5 | 94·4 |
| | Median | | 5·7 | | | | 1·9 | | 6·6 | 3·0 |
| | | 10 of 10 >90th centile | | | 4 of 10 >90th centile | | | 4 of 10 >90th centile | | |
| | | 9 of 10 >95th centile | | | 4 of 10 >95th centile | | | 3 of 10 >95th centile | | |
| 46,XX ($n$=66, median) | | | 1·00 | | | 0·98 | | 2·3 | 0·96 | |
| 46,XY ($n$=76, median) | | | 1·01 | | | 1·01 | | 2·7 | 1·11 | |
| | Median | | 1·01 | | | 1·00 | | 2·6 | 1·03 | |

*Percentage of hCG molecules detected by the H-hCG assay (hCG (ng/ml)÷H-hCG (ng/ml)).

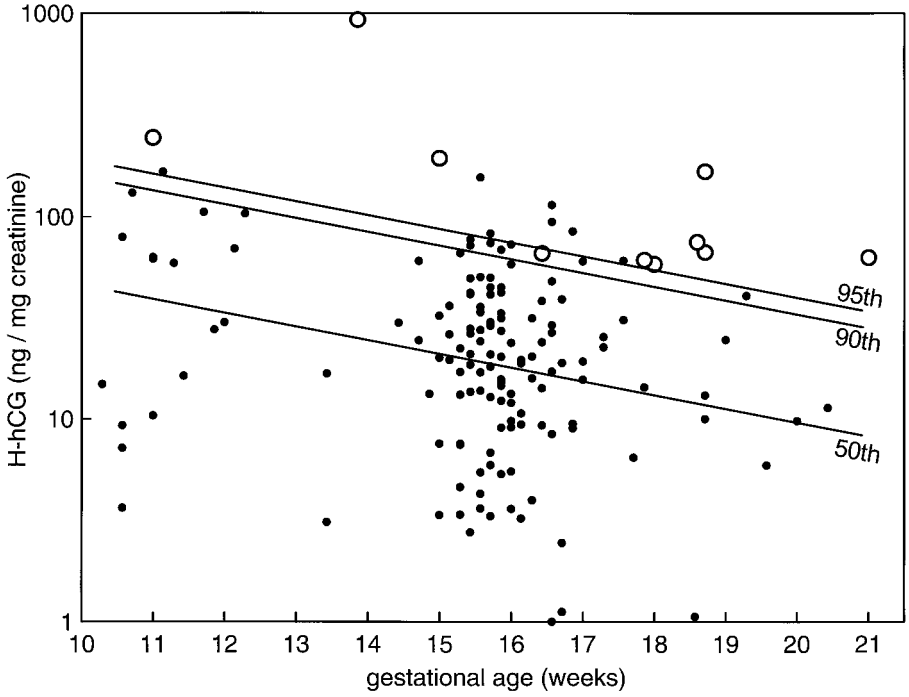


Fig. 2—H-hCG levels (ng/mg creatinine) in 142 normal (●) and 10 Down syndrome (○) pregnancy urine samples

1·03. There was no significant affect of fetal sex on this median. The median MoM for the Down syndrome cases was 3·0, indicating a 3·0-fold increase in the portion of H-hCG molecules. The percentage of H-hCG molecules exceeded the 90th centile of unaffected cases in 4 of 10 Down syndrome cases, and exceeded the 95th centile in 3 of 10 cases (Table I).

## DISCUSSION

H-hCG is the principal hCG-related molecule in patients with gestational choriocarcinoma (Elliott *et al.*, 1997). An immunoassay was developed for measuring H-hCG. Using this assay and an hCG test, we determined the amount of H-hCG in hCG-containing samples. H-hCG accounted for 100 per cent of hCG molecules in choriocarcinoma patient samples. In contrast, it accounted for only 1·6 per cent of hCG molecules in normal first-trimester and 2·7 per cent in second-trimester pregnancy samples. A three-fold increase in the percentage of H-hCG was observed in Down syndrome pregnancy samples (urine).

hCG and H-hCG levels were measured in urine samples from normal and Down syndrome pregnancies. We noted a 1·9-fold increase in hCG levels in Down syndrome over normal cases. In contrast, we noted a 5·7-fold increase in H-hCG levels in Down syndrome over normal cases. Using hCG, 40 per cent of Down syndrome cases exceeded the 90th and 95th centiles of normal pregnancies. Measuring H-hCG, however, all of the 10 cases exceeded the 90th centile, and 9 of 10 cases exceeded the 95th centile of normal pregnancies. The urine hCG screening performance was comparatively poor. It was the same, however, as that noted with serum hCG screening (Canick, 1990). H-hCG screening performance was much better. 90 per cent detection at 5 per cent false-positive rate is higher than has been previously reported for a single screening test. Eight of 10 Down syndrome cases were from the second trimester of pregnancy. Seven of these 8 cases exceeded the 95th centile of unaffected pregnancies. The other two Down syndrome cases were from the 11th and 13th week of pregnancy. Both of these cases exceeded the 95th centile of unaffected pregnancies. The value of the H-hCG test is most

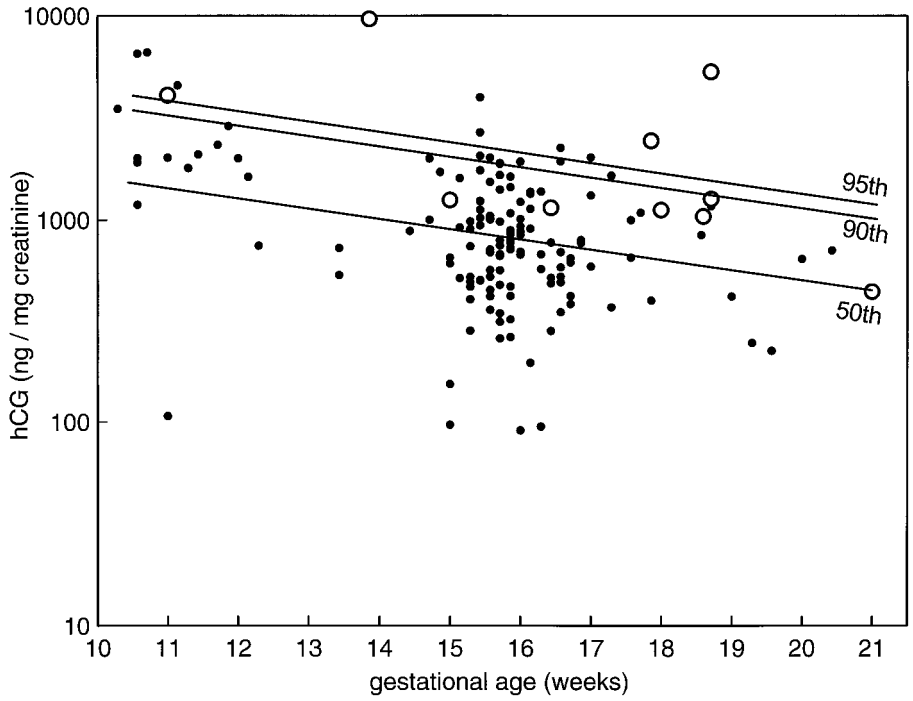


Fig. 3—Extent of hyperglycosylation of hCG. The proportion of H-hCG molecules is calculated (H-hCG (ng/ml)÷hCG (ng/ml)) in 142 normal (●) and 10 Down syndrome (○) pregnancy urine samples

clearly shown in the second trimester of pregnancy. This value is potentially enhanced by the indication that it may also be useful in the first trimester of pregnancy.

This is the first report with the H-hCG assay. The results are preliminary, since they were obtained with a relatively small number of samples. Consequently, the data should be considered with caution, particularly as they relate to first-trimester screening. No clear correlation was noted between fetal sex, maternal age or maternal weight and H-hCG levels (insufficient samples to prove the presence or absence of a relationship). Further studies are clearly warranted to expand upon these preliminary findings, to confirm the high sensitivity for Down syndrome cases, to examine combinations of H-hCG and other screening tests (oestriol, AFP, PAPP-A and nuchal translucency), and to determine the effects of personal parameters (age, weight, etc.) on H-hCG levels. Studies with specimens from choriocarcinoma patients indicate that this test works in serum or urine samples. The screening performance of the test should also be investigated in serum samples.

To consider H-hCG as a Down syndrome screening test practical matters must be contemplated. The stability of H-hCG, storage and shipping conditions, assay adaptability and cost should be considered. When pure hCG (batch P8 from pregnancy urine) and pure H-hCG (batch C3-II from choriocarcinoma patient urine) were added to normal urine and stored for up to four weeks at ambient temperature (22°C), they dissociated slowly. hCG dissociated at a rate of 1·6 per cent per week, and H-hCG at 4·0 per cent per week. H-hCG immuno-reactivity could not be generated from the dissociation or degradation of hCG. Both hCG and H-hCG (in urine) were particularly stable at 4°C (dissociation rate <0·28 per cent per week) (Isozaki and Cole, paper in preparation). Spot urine samples may be collected for H-hCG and creatinine determinations. Considering the stability of H-hCG, samples could be kept for numerous hours at ambient temperature before assay or before refrigeration. To avoid losses,

samples should probably be placed in a refrigerator for extended or multi-day storage. Samples should probably be shipped with ice or refrigerant (blue ice). The H-hCG assay is a rapid, straightforward, enzyme-linked sandwich type test. It could easily be adapted to an automated immunoassay protocol. The cost should be similar to a pregnancy test. H-hCG may be a practical test for Down syndrome screening.

We considered the basis for elevated H-hCG levels in Down syndrome cases. hCG levels are about two-fold elevated in Down syndrome cases. This suggests about a two-fold increase in secretion of hCG in Down syndrome cases, or a delay in the normal decrease of hCG levels in these cases. The percentage of H-hCG rises to three-fold in Down syndrome cases. This may be due to abnormal post-translational processing in Down syndrome trophoblast cells (Cole *et al.*, 1997a). Increased hCG levels and increased hyperglycosylation join together to elevate H-hCG production in Down syndrome cases by about six-fold.

We considered the different pathways that may hyperglycosylate hCG in Down syndrome cases. Processing of hCG oligosaccharides is completed in the golgi apparatus of trophoblast cells. Sialic acid residues terminate the sugar chains. Prior to the addition of sialic acid residues, branching of oligosaccharides can occur and additional sugar chains (antennae) can be added. Additional antennae are present on the N-linked and O-linked oligosaccharides of H-hCG. The availability of sialic acid residues and the activity of the sialyltransferase enzyme may control the hyperglycosylation of glycoproteins. Sialic acid is made from phosphoenolpyruvate, an end product of glucose metabolism. As such, glycosylation may be affected by the activity of the different enzymes involved in glucose metabolism, or by regulators of sugar metabolism, glucose, ATP or oxygen. Abnormal glucose uptake has been observed in choriocarcinoma cells (Hahn *et al.*, 1994). Abnormal glucose uptake and poor vascular supply may explain the high proportion of hyperglycosylated molecules in choriocarcinoma cases. Very small changes in glucose uptake, glycolytic enzyme or oligosaccharide processing enzyme activity could explain the 6·6 per cent hyperglycosylation of hCG observed in Down syndrome pregnancies.

H-hCG is a promising new screening test for Down syndrome. H-hCG could ultimately replace hCG determinations in Down syndrome screening. Measurement of H-hCG may surmount the limitations of hCG and triple test, including the comparatively low sensitivity, the high false-positive rate, the abnormally high licensing fees, and possibly the non-applicability to first-trimester samples. The ease and convenience of a urine test further expands the potential appeal of this test.

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