by a specific method, such as LC-MS. It is also possible that differences in the calibration of the 2 assays could contribute to this apparent improvement.

These results confirm that although immunoassay-measured DHEAS interferes less in the Roche Testosterone II assay than in the Testosterone assay, the Testosterone II assay still appears susceptible to interference from DHEAS or by other steroids detected by the DHEAS immunoassay. This work highlights residual specificity problems with the Roche Testosterone II assay and provides further evidence of the problems in developing direct testosterone immunoassays with a specificity adequate for measuring testosterone in women.

**Author Contributions:** All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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**Fig. 1.** The difference between Roche Testosterone/Testosterone II assay measurements and LC-MS testosterone measurements plotted against DHEAS concentration.

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**References**


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To the Editor:

Hyperglycosylated human chorionic gonadotropin (hCG-h) is the major form of hCG occurring in early pregnancy and trophoblastic disease, whereas the glycans are smaller in hCG produced later in pregnancy (1, 2). hCG-h can be measured by immunoassays based on monoclonal antibody B152, which reacts with a type 2 core

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Fig. 1

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**Letters to the Editor**

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Elimination of Complement Interference in Immunoassay of Hyperglycosylated Human Chorionic Gonadotropin

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O-glycan attached to Ser132 and surrounding peptide structures but not with hCG lacking this glycan or having a type 1 core O-glycan in this position (3). In early pregnancy, reduced hCG-h concentrations in urine have been associated with early pregnancy loss (2), whereas increased concentrations at 9 to 12 weeks have been associated with Down syndrome (1). A high proportion of hCG as hCG-h is useful in diagnosing malignant trophoblastic disease (4). These results need to be confirmed, but an earlier-version assay for hCG-h is not presently available (5). We developed a time-resolved immunofluorometric assay that uses B152 and found that serum contains factors that interfere with the assay to cause falsely low results. We demonstrated B152 to be an IgG2a antibody (Mouse Monoclonal Isotype Kit; Serotec, http://www.abdserotec.com), which interacts with complement to decrease its binding capacity. This interaction can be prevented with calcium chelators or by heating serum (6). We used EDTA to eliminate the interference.

The sandwich immunoassay for hCG-h was performed via a 2-step incubation protocol in 96-well plates, essentially as previously described, but we used monoclonal antibody B152 (3) as the capture antibody, and as the tracer, we used a Eu chelate–labeled in-house monoclonal antibody (1B2) to the β subunit of hCG (hCGβ). hCG-h produced by cultured JEG-3 choriocarcinoma cells was used as the calibrator. We used the time-resolved dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA®; PerkinElmer-Wallac) to measure the hCG concentration in JEG-3 medium and used that result to assign hCG-h values. DELFIA measures intact hCG but not the free β subunit (7, 8). A calibrator containing 8.8–8800 pmol/L of hCG-h or sample (50 µL) and 150 µL assay buffer were incubated for 2.5 h in the antibody-coated wells. The wells were washed, 100 ng of Eu-labeled tracer antibody in 200 µL buffer was added, and the plates were incubated for 0.5 h. The plates were washed again, and the fluorescence was measured by time-resolved fluorometry (8). The limit of detection was 2 pmol/L, and the limit of quantification was 5 pmol/L. The CV was <10% at concentrations >10 pmol/L. The response curve was linear over the measurement range.

Samples of serum, plasma, and urine were obtained from 8 women during pregnancy weeks 4 to 6. Leftover samples from 20 men and 20 nonpregnant women were used to study the recovery of hCG-h that had been spiked into serum and plasma to a concentration of 22 000 pmol/L. The pregnant women who provided samples gave informed consent. Disodium EDTA was added to serum to a final concentration of 5 mmol/L, which corresponds to that in EDTA-containing plasma. The samples were analyzed after 1–20 h.

The median hCG-h concentration in serum samples from pregnant women was 33% (range, 22%–46%) of that in EDTA-containing plasma, and the recovery was 16% to 38% in serum spiked with hCG-h.

Diluting the serum with diluent to 1 part in 100 increased the recovery to 103% of that in undiluted EDTA-containing plasma. Higher dilutions did not increase recovery further. At a 10-fold dilution, recovery was 80%–100%. Dilution of plasma by 100-fold caused a 6% increase in recovery (P = 0.020). The addition of EDTA to serum increased recovery to 95% of that in undiluted plasma (P = 0.008; Table 1). Dilution of urine with assay buffer had no effect on recovery. If serum was first added to the assay well and EDTA-containing buffer was added afterward, the recovery of hCG-h was about 15% lower than if the serum sample was first diluted with EDTA-containing buffer before it was added to the well. Heating of serum at 56 °C for 60 min also eliminated the interference but also caused some dissociation of hCG.

At 4 to 5 weeks of pregnancy, virtually all (90%–100%) of the hCG in serum samples consisted of hCG-h. The content decreased to 5%–10% at 10 weeks and to <3% after 20 weeks. In urine, the proportion of hCG-h at 4 to 5 weeks was 100%–120%. These results agree with those obtained by the original method (2).

The problem of complement with the use of B152 for assay of hCG-h in serum has not previously been described. A report on an ear-

### Table 1. Recovery of 22 000 pmol/L of hCG-h spiked into 20 samples each of EDTA-containing plasma and serum. a

<table>
<thead>
<tr>
<th></th>
<th>Mean, %</th>
<th>SD, %</th>
<th>CV, %</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Serum with EDTA</td>
<td>88.8</td>
<td>10.0</td>
<td>11</td>
<td>0.008</td>
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<tr>
<td>Serum (100-fold dilution)</td>
<td>103.0</td>
<td>12.3</td>
<td>12</td>
<td>0.362</td>
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<tr>
<td>Plasma</td>
<td>93.7</td>
<td>13.3</td>
<td>14</td>
<td>0.008</td>
</tr>
<tr>
<td>Plasma (100-fold dilution)</td>
<td>100.0</td>
<td>14.4</td>
<td>14</td>
<td>Reference</td>
</tr>
</tbody>
</table>

* The results for EDTA-containing plasma diluted to 1 part in 100 were defined as 100%. The P values denote a difference in recovery relative to that of EDTA-containing plasma diluted 100-fold. The recovery of hCG-h in serum supplemented with 5 mmol/L EDTA was 5% lower than in undiluted EDTA-containing plasma (P = 0.016).
lier commercial assay, which also used B152 as the capture antibody, stated that diluting serum samples produced the expected results and that results for serum and plasma were identical (5). This assay has been used in several clinical studies. It remains to be seen whether earlier promising results on the clinical use of hCG-h can be verified and possibly improved by eliminating the variable effect of complement in serum.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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The Detection of Double Mutations in KRAS Depends on the Mutation-Detection Assay Used

To the Editor:

Epidermal growth factor receptor is frequently overexpressed in colorectal cancer, and targeted therapies have been developed to block this receptor. Monoclonal antibodies such as cetuximab and panitumumab have demonstrated effectiveness in increasing the survival rates for some metastatic patients. Patients with mutations in codon 12 or 13 of the KRAS (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) gene are not responsive to this therapy, however, and tumors from patients are systematically screened for somatic KRAS mutations before treatment. Several techniques have been developed to screen for KRAS mutations, and the differences in these techniques with respect to diagnostic sensitivity, diagnostic specificity, and efficiency for mutation detection have been described (1–4). The main potential weakness of direct sequencing is its low diagnostic sensitivity (approximately 20%). Other techniques, such as TaqMan® PCR-based assays and SNaPshot® (Applied Biosystems), have diagnostic sensitivities between 1% and 10%. In this study, we analyzed 1970 colorectal carcinoma tumors for KRAS mutations by direct sequencing. We detected 10 cases with double mutations at codons 12 and 13 via direct sequencing and compared the ability of single-nucleotide extension (SNE) and hydrolysis probe real-time PCR to detect these rare mutations. The nucleotide pairs implicated in the nucleotide changes are 34 and 35, 35 and 36, 35 and 37, 35 and 38, 37 and 38, and 38 and 39. Sequencing analysis did not allow us to determine whether the 2 mutations were located on the same allele.

With hydrolysis probe real-time PCR, we detected both mutations in only 1 case (c.35G>T and c.38G>A); 9 of the tumors were classified as wild type for codons 12 and 13. The lack of detection of either one of the double mutations implies that the 2 mutations are located on the same allele. Yet, the detection of mutations c.35G>T and c.38G>A suggests that these mutations are located on separate alleles, although the c.35G>T mu-