Evaluation of Four Commercially Available Assays for Free Thyroxin

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We assessed two step-by-step and two analog assays for measuring free thyroxin (FT4) in serum: Clinical Assays' "GammaCoat Free/Total T4, (CA), Vitek's "KineticCount Phase II Free T4" (VTK), Diagnostic Products Corporation's "Coat-a-Count Free T4" (DPC) kit (June 1987 version), and Amersham's "Amerlex-M Free T4" (AMX). The VTK assay is automated except for the initial pipetting step. Interassay results correlated well except for samples with abnormal serum albumin concentrations. FT4 values for hypoalbuminemic samples showed a highly significant (P < 0.001) correlation with serum albumin concentration in the DPC and AMX assays. The relationships are described by the equations y = 0.382albumin (g/L) + 0.81 pmol/L and y = 0.450albumin (g/L) - 3.20 pmol/L, respectively. When we used an equation derived from the Law of Mass Action to adjust FT4 values to values expected at an ideal albumin concentration, the observed correlation of albumin and FT4 was abolished completely in the DPC assay, and partly so in the AMX assay. The precision of CA was comparable with that of the analog assays; the CV for the VTK assay was approximately twice that for the other three assays.

Serum thyroxin (T4) is the analyte most frequently measured in assessing thyroid status.5 The major problem in its clinical use is the effect of thyroxin-binding globulin concentration on the total T4T concentration. Direct and indirect assessments of thyroxin-binding globulin concentration are frequently used to overcome this problem, but they are cumbersome and inaccurate at extreme concentrations. Measurement of free T4 (FT4) by equilibrium dialysis has been a research tool, but it is too labor intensive for use in routine clinical analysis. Of the earlier commercial radioimmunoassays for FT4 based on chromatographic separation—microencapsulation, complex kinetic rate, and two-step immunoextraction (T)—only the last method remains. The two-step method is sensitive to methodological variations: temperature, pipetting time, and degree of mixing. Hence precision is a problem. With the introduction of one-step analog assays, precision was considerably improved. However, the initially developed assays showed marked dependence on albumin concentration and the presence of albumin-bound substances (2–7). Commercial suppliers have tried to overcome this by (a) chemically inhibiting analog—albumin binding, (b) adding albumin to buffer solutions, and (c) optimizing analog and antiserum concentrations (6, 9).

Our aim here was to evaluate a newly introduced two-step FT4 assay, "Phase II," that involves the use of an automated RIA system, "KineticCount48" (Vitek Systems, Hazelwood, MO 63042), and two analog methods, "Coat-a-Count FT4" (Diagnostic Products Corp., Los Angeles, CA 90045) and "Amerlex-M Free T4" (Amersham plc., Amersham, Bucks., U.K.), comparing these with the two-step assay we currently use, "GammaCoat FT4," (Clinical Assays, Cambridge, MA 02139). The manufacturers of the analog assays claim that further improvements have totally eliminated the "albumin artefact" (8, 10).

Materials and Methods

The 183 patients' sera used in the study were chosen without conscious bias from among those submitted for routine thyroid-function tests. Not all assays were performed on every patient, owing to insufficient sample volume. Triiodothyronine (T3) was measured with the "Amerlex-M T3 RIA" (Amersham plc.) and thyrotropin (TSH) was measured with "TSH MAIAclone" (Serono Diagnostics Ltd., Woking, Surrey, U.K.). The respective reference intervals were 0.91–2.91 nmol/L and 0.4–3.8 milli-int. units/L.

In addition, we studied 40 sera from hospitalized patients with low serum albumin concentrations (17–30 g/L). All patients in this group were clinically euthyroid and could be classified as having nonthyroidal illness; 12 patients died within a month of sampling. All patients had at least one of the following conditions: metastatic cancer (13 patients), chronic renal failure (10 patients, three had had recent transplants), severe infection or inflammatory disease (14 patients), chronic liver disease (seven patients), congestive heart failure (five patients), acute myocardial infarction (three patients), and immediate postoperative (three patients). In addition, 13 of these patients had received intravenous heparin. TSH was measured in the serum of these patients with the "Echolonal-hTSH" kit (Bio-Rad Labs., Anaheim, CA 92806), for which the reference interval is 0.23 to 5.41 milli-int. units/L.

We measured serum albumin by the bromcresol green dye-binding method as automated in the BM/Hitachi 737 Clinical Chemistry Analyzer (Boehringer Mannheim, Montreal, Canada), with reagents supplied by the manufacturer. In our laboratory, the reference interval for albumin is 34–46 g/L.

"GammaCoat FT4" (CA). All assays were performed as recommended by the manufacturer. The radioactivity in the tubes was counted for 60 s in a Model 1280 Multigamma II gamma counter (LKB Wallac, Turku, Finland). Results
were derived from the standard curve by cubic spline data analysis. The reference interval supplied by the manufacturer from data on 846 euthyroid hospitalized patients is 7.7–27.0 pmol/L.

"Phase II Assay" (VTK). All assays were performed as recommended by the manufacturer. Operator involvement was limited to pipetting of samples (quality controls or standards), incubation buffer, and 125I-labeled tracer buffer. The rest of the procedure was fully automated with the "KinetiCount48." Results were subjected to Rodbard curve analysis. The system is able to use stored standard curves, which are valid for two weeks. The supplier's stated reference interval for hospitalized patients is 11.0–23.0 pmol/L.

"Coat-a-Count FT₄⁺ (DPC). All assays were performed as suggested by the manufacturer. Radioactivity was counted for 60 s in a Model 4/600 gamma counter (Micromedic Systems, Horsham, PA 19044). Data reduction was done in the log-logit mode. The supplier's quoted reference interval for euthyroid patients is 10.3–25.7 pmol/L.

"Amerlex-M FT₄⁺ (AMX). All assays were performed as suggested by the manufacturer. Radioactivity was counted in a Model 4/600 gamma counter, and data were subjected to cubic spline data analysis. The reference interval established by the manufacturer is 9.0–25.0 pmol/L.

The between-run CV was calculated on results obtained by assaying three concentrations of "Lyphochek Human Control Serum" (Bio-Rad Labs.): master lot no. 30000 for CA and VTK, and master lot no. 17500 for DPC and AMX.

We analyzed the differences in precision between the assays for significance by the F-test (ratio). Linear regression analysis was used to study the albumin effect on FT₄⁺ measurements. The significance of the correlation was determined by Student's two-tailed t-test.

As analog FT₄⁺ values appeared to be lower than values by two-step assay in hypoalbuminemic patients, the analog FT₄⁺ values were adjusted to what they would be if the albumin concentration in serum was 42 g/L (the concentration of albumin in AMX standards, and the mean albumin concentration in the normalalbuminemic group of patients) by the following formula, derived from the Law of Mass Action (11):

\[
F_{\text{corr}} = \frac{[F_{\text{meas}} - \left(\frac{([\text{Alb}]/42 - 1)}{K_{\text{An}}}\right)] 	imes 42}{[\text{Alb}]}
\]

where \(F_{\text{corr}}\) = corrected FT₄⁺, \([F_{\text{meas}}]_{[\text{Alb}]}\) = albumin mass concentration, \(K_{\text{An}}\) = affinity constant for analog–antibody binding; \(K_{\text{An}}\) is considered to be 15 L · pmol⁻¹ (12).

Results

Precision

Between-run CVs for the analog assays were <8% at all three quality-control concentrations. There was no significant difference between results by the two assays. With the two-step assays, CA had significantly lower CVs (\(P < 0.025\)) than did the VTK assay. At higher quality-control concentrations, there was no significant difference between CA and the analog assays (Table 1). With VTK, the reproducibility of results from two separate runs was poor (Table 2); on four occasions, the difference was >10 pmol/L.

Effect of Low Serum Albumin

Both analog assays demonstrated markedly lower FT₄⁺ concentrations in sera with low albumin concentrations (Figure 1); this was not observed in the two-step assays (Figure 2). Studies on correlation of FT₄⁺ (y) with serum albumin concentration (x) gave r values of 0.59 and 0.69 (\(P < 0.0001\)) for DPC and AMX, respectively. The slopes of the linear regression lines for DPC and AMX were 0.382 and 0.450, respectively, whereas the slope for CA was 0.085 and that for VTK was 0.006. After adjusting the analog FT₄⁺ values for serum albumin concentration, the slope for the DPC assay became −0.010, whereas the slope of AMX was 0.136.

We divided all patients into two groups according to their serum albumin concentration. The mean FT₄⁺ in the hypoalbuminemic group (serum albumin <34 g/L) was significantly lower (\(P < 0.0005\)) than the normalalbuminemic group for both analog assays (Table 3). When these means were recalculated after adjusting each patient's FT₄⁺ concentration by the above formula, there was a significant difference between the groups for AMX only.

Clinical Accuracy

In normoalbuminemic patients, interassay results correlated well (Table 4). These patients were classified as: (a) hypothyroid if their TSH exceeded one assay SD above the upper quoted reference limit, (b) hyperthyroid if their T₃ exceeded one assay SD above the upper limit and TSH was below the lower limit of normal, or (c) euthyroid. Thyroid status could not be defined clearly by the above criteria in 40 patients; these patients were excluded from this part of the study. As judged from use of the manufacturers' reference intervals, all but one of the hyperthyroid patients had increased FT₄⁺ concentrations as measured by all four assays (Figure 3). In the euthyroid group, six of 69 and one of 72 patients had subnormal values by VTK and AMX, respectively. For the hypothyroid group, most patients had low-normal values for FT₄⁺ consistent with the diagnosis of subclinical hypothyroidism. VTK showed the largest number of values below the lower reference limit, 10 of 22, compared to five of 23 (DPC), three of 23 (AMX), and two of 24 (CA) for the other assays. With the reference interval established by our laboratory for CA, 11.0–25.0 pmol/L (13), four patients had subnormal FT₄⁺.

In the 40 hypoalbuminemic nonthyroidally ill patients, all were euthyroid as judged by clinical chart review. These patients were classified according to their TSH concentration. One patient with undetectable TSH had a marginally

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**Table 1. Between-Run CV for FT₄⁺ Determined with Control Sera**

<table>
<thead>
<tr>
<th></th>
<th>CA</th>
<th></th>
<th>VTK</th>
<th></th>
<th>DPC</th>
<th></th>
<th>AMX</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Level</td>
<td>n</td>
<td>Mean</td>
<td>CV, %</td>
<td>n</td>
<td>Mean</td>
<td>CV, %</td>
<td>n</td>
<td>Mean</td>
</tr>
<tr>
<td>I</td>
<td>46</td>
<td>5.59</td>
<td>11.4</td>
<td>36</td>
<td>6.32</td>
<td>24.3</td>
<td>20</td>
<td>4.35</td>
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<tr>
<td>II</td>
<td>40</td>
<td>16.78</td>
<td>6.4</td>
<td>31</td>
<td>13.30</td>
<td>12.6</td>
<td>20</td>
<td>13.34</td>
</tr>
<tr>
<td>III</td>
<td>30</td>
<td>64.68</td>
<td>5.3</td>
<td>20b</td>
<td>55.09</td>
<td>12.8</td>
<td>14</td>
<td>47.94</td>
</tr>
</tbody>
</table>

*Lyphochek Human Control Serum, master lot no. 30000 for CA and VTK, and lot no. 17500 for DPC and AMX. Mean values are in pmol/L.

*Values exceeding the highest standard concentration were not included in calculations.*
increased FT$_4$ with CA (25.4 pmol/L) and VTK (24.3 pmol/L) and low-normal values with DPC (11.8 pmol/L) and AMX (9.6 pmol/L). Two patients had above-normal TSH concentrations, 14 and 6.6 milli-int. units/L. Both had normal values for FT$_4$ by CA and VTK, low-normal FT$_4$ by DPC, and a clearly subnormal value with AMX. In the remaining 37 patients with normal TSH concentrations, subnormal FT$_4$ concentrations were found in 20 (AMX), 18 (DPC), and two (VTK) patients. There were no subnormal FT$_4$ values by CA. Two patients had an above-normal FT$_4$ by CA and VTK.

Discussion

Our aim was to determine which commercial FT$_4$ assay is the most suitable for a laboratory servicing a tertiary-care hospital, taking into consideration technical ease, precision, and clinical accuracy. Analog assays are technically simple, but initial development assays showed albumin dependence. With design changes in June 1987, the supplier of DPC claims this effect has been eliminated. The two-step method does not have this "albumin artefact," but it suffers from poor precision, which should improve with automation.

The semi-automated VTK system offered the simplest operation; the manual two-step procedure of CA was the most technically demanding. The precision of the analog methods was not significantly improved over CA. Our laboratory performs the CA assay under strictly controlled technical conditions; comparable precision may not be achieved by all laboratories. The poor reproducibility with VTK was surprising. The imprecision associated with two-step assays has been attributed mainly to technical inconsistencies and variations in ambient laboratory temperature. By automating the assay after the initial pipetting steps and maintaining a constant temperature throughout the analysis, better precision was expected. The stored-curve function of the VTK instrument appeared valid. However, inconsistent readings of the paired standard in runs involving use of stored standard curves resulted in poor precision. The incidence of spurious values was unacceptable.

All assays correlated well in normoalbuminemic patients. In hypoalbuminemic patients with nonthyroidal illness, there was discordance between the two-step and analog assays. There is much controversy over which FT$_4$ method gives the most nearly accurate results in patients with nonthyroidal illness (9, 11). Many factors interplay in these patients to give abnormal accurate results: suppressed TSH production (14, 15), decreased peripheral conversion of T$_4$ to T$_3$ (16), decreased or increased thyroxin-binding globu-

Table 2. CVs for Between-Run Duplicates

<table>
<thead>
<tr>
<th></th>
<th>CA</th>
<th>VTK</th>
<th>DPC</th>
<th>AMX</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of pairs</td>
<td>144</td>
<td>144</td>
<td>98</td>
<td>98</td>
</tr>
<tr>
<td>CV, %, for duplicates</td>
<td>6.7</td>
<td>14.2</td>
<td>7.0</td>
<td>5.4</td>
</tr>
</tbody>
</table>

Fig. 1. Correlation between serum FT$_4$ concentrations as measured by analog assays (left), after adjustment by equation in text (right), and serum albumin concentrations

n = 140 for DPC, 137 for AMX. Parallel lines represent the upper and lower limits of the reference intervals. Note the reclassification of many patients into euthyroid range after adjustment.

2304 CLINICAL CHEMISTRY, Vol. 34, No. 11, 1988
lin and prealbumin (transthyretin), decreased albumin (17), presence of inhibitors of protein binding such as unsaturated nonesterified fatty acids and others, as yet unidentified, whose effects may be pH dependent (16–18), antithyroxiin autoantibodies (19), and drugs (20). The main interferences with the first-generation analog assays were hypoalbuminemia and the presence of albumin-binding inhibitors. DPC claims that binding of analog to endogenous albumin has been totally eliminated in their current assay by the use of two chemical blockers. The combination of blockers gives the lowest analog–albumin binding and no displacement of T₄ from thyroxin-binding globulin (8). They substantiated their claim experimentally by adding up to 50 g of charcoal-adsorbed human serum albumin per liter to third-trimester pregnancy serum. This caused no change in measured F₄ concentration (10). Theoretically, the measured F₄ concentration will remain constant if the decreased F₄ concentration that resulted from increased T₄–albumin binding is equal to the overestimation of F₄ ascribable to analog sequestration by the added albumin. But charcoal-adsorbed human albumin may not have binding properties similar to those of endogenous albumin. Studies have shown that albumin structure and ligand-binding (including T₄) is affected by the number of fatty acid moieties bound per albumin molecule (21, 22).

Amersham also claims that modifications in the AMX assay have minimized the "albumin effect." The assay now contains bovine albumin in the buffer solution (9) to compensate for decreases in serum albumin concentrations and to sequestrate nonesterified fatty acids. Eknes (11), in a review of the binding characteristics of currently available analogs, contends that using blockers to eliminate analog–albumin binding completely will magnify the effect of thyroxin-binding globulin concentration on F₄ measurement; and the presence of endogenous or exogenous binding competitors, while increasing analog availability, also results in increased free hormone concentration.

The other argument supporting the accuracy of analog F₄ assays is based on reports of low F₄ concentrations, as determined by equilibrium dialysis and calculation methods, in patients with nontyroidal illness and pregnancy (23–28). These patient groups tend to give lower F₄ values with analog methods. True hypothyroxinemia occurs in a subgroup of patients with nontyroidal illness (14, 15, 23), but less severely and less frequently than analog assays suggest (4, 5, 29–32). Furthermore, equilibrium dialysis, although the reference method for F₄ analysis, can be affected by some of the changes mentioned above for patients with nontyroidal illness. Indirect equilibrium dialysis methods involve multiplying the percent dialyzable fraction by the total or adjusted T₄ to obtain the F₄ value. In low-T₄ patients with nontyroidal illness, total T₄ and adjusted T₄ values are often low, owing to decreased binding protein concentrations and interferences with resin uptake tests by endogenous inhibitors (16, 18) In the direct equilibrium dialysis methods, in which F₄ in the dialysate is measured by radioimmunoassay, spurious results may occur owing to dilution effects. For samples with normal-binding protein concentrations, up to 60-fold dilutions do not affect

![Graph](image.png)

**Fig. 2. Correlation between serum F₄ concentrations as measured by two-step assays and serum albumin concentrations**

- **n** = 148 for CA, 132 for VTK. Parallel lines represent the upper and lower limits of the reference intervals.

### Table 3. Mean of Measured and Adjusted FT₄ in Nomoalbuminemic and Hypoalbuminemic Patients

<table>
<thead>
<tr>
<th></th>
<th>Nomoalbuminemic</th>
<th>Hypoalbuminemic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measured</td>
<td>Adjusted</td>
</tr>
<tr>
<td>DPC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>98</td>
<td>42</td>
</tr>
<tr>
<td>FT₄, pmol/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>17.14</td>
<td>17.15</td>
</tr>
<tr>
<td>SD</td>
<td>3.83</td>
<td>3.90</td>
</tr>
<tr>
<td>Albumin, g/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>42.4</td>
<td>27.5</td>
</tr>
<tr>
<td>AMX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>98</td>
<td>39</td>
</tr>
<tr>
<td>FT₄, pmol/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>16.03</td>
<td>18.62</td>
</tr>
<tr>
<td>SD</td>
<td>3.47</td>
<td>4.00</td>
</tr>
<tr>
<td>Albumin, g/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>42.4</td>
<td>27.3</td>
</tr>
</tbody>
</table>

*FT₄ concentration adjusted for albumin concentration by use of the equation given in the text.

**Table 4. Interassay Correlation of FT₄ Concentrations in Nomoalbuminemic Patients**

<table>
<thead>
<tr>
<th>x</th>
<th>y</th>
<th>n</th>
<th>Slope</th>
<th>Intercept</th>
<th>r</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA</td>
<td>VTK</td>
<td>143</td>
<td>0.93</td>
<td>0.53</td>
<td>0.94</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CA</td>
<td>DPC</td>
<td>144</td>
<td>0.77</td>
<td>3.91</td>
<td>0.92</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CA</td>
<td>AMX</td>
<td>144</td>
<td>0.90</td>
<td>0.53</td>
<td>0.94</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AMX</td>
<td>DPC</td>
<td>146</td>
<td>0.81</td>
<td>4.23</td>
<td>0.93</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Significance of correlation between x and y.
FT$_4$ concentration (33, 34). However, in samples with low binding-protein concentrations, decreasing FT$_4$ concentrations may be apparent at 16-fold dilution (34). In addition, concomitant dilution of inhibitors may result in lowered FT$_4$ concentration through increased hormone binding (17).

Evaluation of FT$_4$ methods for patients with nonthyroidal illness is hampered by the lack of a "gold standard" for assessment of thyroid status in these patients. Even ultrasensitive TSH assays have proved inadequate in many patients (37, 38).

In many studies, direct equilibrium dialysis and two-step methods seem to give the highest proportion of "correct" values (4, 5, 16, 29-32, 35, 36). Similarly, in our series, the two-step assay values correlated better with thyroid status as assessed by other thyroid function tests or clinical review, or both. For the hypoalbuminemic patients with nonthyroidal illness there were only two normal FT$_4$ values with VTK, and none with CA. Even in the two patients with above-normal values for TSH, the normal FT$_4$ results seen with the two-step assays may be more nearly accurate, because marginally increased TSH concentrations may exist in some patients with nonthyroidal illness (16, 18, 39).

We demonstrated a highly significant correlation of analog FT$_4$ values with albumin concentration. Furthermore, with the DPC method, the mean FT$_4$ of the hypoalbuminemic group increased to the same value as the normoalbuminemic group after each sample result was adjusted to the "ideal" albumin concentration, 42 g/L, with an equation derived from the Law of Mass Action. The assumptions made are that the analog binds only to albumin and that the analog–albumin binding constant remains the same throughout the range of albumin concentrations. With AMX, the same equation failed to normalize the mean FT$_4$ completely, implying that not all assumptions were met. This adjustment factor cannot be used for the two-step assays because there is no contact of serum proteins with the tracer.

It has been suggested that the correlation seen between albumin concentration and FT$_4$ values in patients with nonthyroidal illness is fortuitous, and is explained by FT$_4$ and albumin independently correlating with the severity of illness to the same degree, and that the discrepancy between analog and nonanalog methods is the result of overestimation of FT$_4$ by the latter methods (40). Nonesterified fatty acids have been suggested as the interferant in equilibrium dialysis methods, because long incubation results in significant nonesterified fatty acid generation and displacement of T$_4$ from binding proteins (41). However, two-step assays have shorter incubation periods than do analog methods, but they show the same increase in FT$_4$. In situations where nonesterified fatty acid is generated by increased in vivo or in vitro lipolytic activity, an actual increase in FT$_4$ occurs before sample analysis and should cause increased values in analog methods as well. Wilkins and Midgley (40) state that the bovine albumin in the AMX buffer acts as a sequestator of nonesterified fatty acid, minimizing its effect. However, the DPC kit, which does not contain bovine albumin, gave FT$_4$ results similar to those obtained by AMX in our study.

Analog assays probably give lower FT$_4$ values in hypoalbuminemic patients with nonthyroidal illness as compared with two-step assays. This discrepancy could result from the previously described "albumin effect." Alternatively, it could be caused by the presence of a factor that selectively interferes with thyroxin–antibody binding in the two analog assays but not in the two-step assays, and does not affect analog–antibody binding. This latter possibility is unlikely, because antibodies for analog and two-step FT$_4$ assays should have similar T$_4$ specificity and, theoretically, the antibody must have similar binding characteristics for the analog and native T$_4$.

Even CA showed some effect of albumin on FT$_4$ concentrations, perhaps related to the nature of the antibody used in CA. The current assay is made for measuring both FT$_4$ and total T$_4$, and the kit contains high-avidity antibody for the latter, which may "strip" T$_4$ from binding proteins. Clinically, this did not pose a problem for the interpretation of FT$_4$ in our patients.

Midgley et al. (9) argued that thyroid-function tests are contraindicated in patients with nonthyroidal illness. In our laboratory, 25–30% of all requests for FT$_4$ are for hospitalized patients. The question of thyroid dysfunction often arises with ill patients, requiring prompt diagnosis and treatment. On the basis of the above discussion, we believe that two-step assays are more reliable for these patients. They have the advantages of less sample dilution and no contact between either serum proteins or protein-binding inhibitors and the tracer, so that tracer binding to the antibody is unaffected by binding protein or total hormone concentrations in the sample. The analog methods gave lower FT$_4$ values in hypoalbuminemic patients with nonthyroidal illness, despite the lack of albumin effect on the assay in vitro.

We thank Vittek Systems for providing the KinetiCount48, Diagnostic Products Corp. for Coat-a-Count FT$_4$ kits, and Amersham for the Amerlex-M FT$_4$ kits.

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