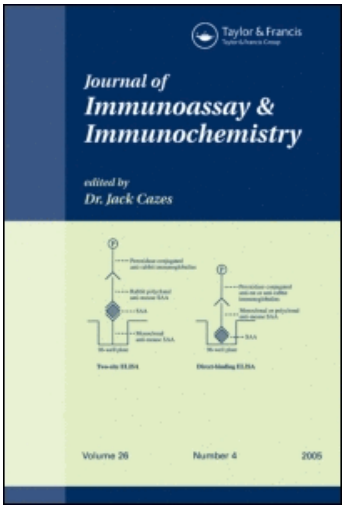

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TIME-RESOLVED IMMUNOFLUOROMETRY OF SERUM HTSH WITH ENHANCED SENSITIVITY

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TIME-RESOLVED IMMUNOFLUOROMETRY OF SERUM hTSH WITH ENHANCED SENSITIVITY

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ABSTRACT

Sensitive TSH immunoassays offer a clear advance in discriminating the TSH concentrations in serum between hyperthyroid and euthyroid individuals; they have been proposed as the best single screening test for thyroid disorders. We have developed a highly sensitive serum TSH TRFIA based on DELFIA technology. Three monoclonal antibodies (McAbs) directed against different epitopes of the TSH molecule were involved in this assay, of which, one McAb was used to coat clear microwells, and the other two were biotinylated for signal generation after being bound by the europium labeled streptavidin. The europium label captured on the well surface was quantified by a routine dissociation-enhancement procedure. The fluorescence intensity was directly proportional to the serum hTSH concentration. The assay required two steps and could be completed within 5 h. The analytical sensitivity reached 0.002 mIU/L with a sample volume of

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100 μL, the function sensitivity was 0.017 mIU/L. Measurements by the present method correlated well with that obtained by the ACS-180 chemi-luminescence immunoassay (CLIA). The discrimination of hyperthyroid patients from clinically euthyroid patients by the present method was much better than that by the routine IRMA.

INTRODUCTION

Improving the analytical sensitivity of immunoassay has been a continuous interest for many clinical chemists.(1–4) Highly sensitive immunoassays can be applied for a variety of applications,(5) such as the measurement of analytes at sub-normal low concentrations, detection or identification of new biologically significant substances, measurement of antigens or antibodies to infectious agents, determination of the receptor numbers on cell surfaces, etc. Current immunoassays in which sensitivity has been of particular concern mainly include thyrotropin in the diagnosis of hyperthyroidism,(6) prostate-specific antigen in early detection of cancer recurrence,(7) HIV antigen or anti-HIV antibody in diagnosis of AIDS,(8,9) and luteinizing hormone in differentiation of normal male prepuberty and hypogonadotrophic hypogonadism.(10) Future expansions to this analyte menu will further increase the demands on analytical sensitivity.

Additionally, immunoassays with enhanced sensitivity make it possible to use urine or oral fluids as samples to monitor therapeutic drugs and hormones or to detect antibodies to various viruses, although these analytes are present in urine or oral fluids, usually at a much lower level than in blood. It also can be expected that sensitive immunoassay will benefit shortening the incubation time and makes miniaturization of immunoassay more accessible.

Accompanying the availability of highly specific McAbs and highly detectable non-isotope labels, immunoassays in immunometric mode have enabled reliable measurement of many analytes beyond the concentration of 10^{-15} mol/L.(11,12) Up to now, many kinds of non-isotope labels have been extensively studied and applied in immunoassays, and all these techniques can achieve a high sensitivity. However, immunoassay based on fluorescence detection is regarded as potentially the most sensitive one because of the excellent delectability of fluorescent label, of which the ultimate sensitivity, detection of a single molecule, has been repeatedly demonstrated with fluorometric analysis.(13) However, only after the introduction of time-resolved fluorescence immunoassay (TRFIA), where lanthanide ions or their ligands are employed as labels, has TRFIA become a routine technology

for supersensitive immune-analysis because of the elimination of various sources of background fluorescence.

Even though assaying TSH alone isn't enough for evaluation of thyroid functions, TSH assays with enhanced sensitivity permit a more reliable differentiation between euthyroid and hyperthyroidism subjects, and also was useful in monitoring TSH levels in thyroid carcinoma patients under thyroxin treatment.(14) Various strategies have been employed to improve both the analytical and functional sensitivity of TSH immunoassay, such as multiple labeling,(15,16) signal amplification,(15,17) adopting a more efficient separation technique,(18) and carefully optimizing the assay systems,(19) etc.

Herein, we describe a sensitive time-resolved immunofluorometric assay (TR-IFMA) for serum TSH, based on double detection antibodies and streptavidin-biotin amplifications. The simultaneous binding of the two detection antibodies allowed approximately 1.5- and 2.5-times increase of fluorescence response, compared to that when single detection antibodies were used; the streptavidin-biotin interaction further amplified the specific fluorescence signals. When carefully optimized, the background fluorescence can be controlled at a low level, generally less than 1500 CPS.

The present method has an analytical sensitivity of 0.002 mIU/L and exhibits better performance for separating hyperthyroid from euthyroid patients, compared with IRMA. A schematic diagram of the final complex captured on the microwell surface is shown in Figure 1.

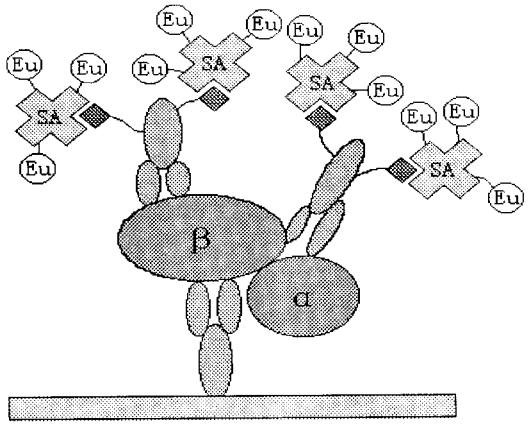

Figure 1. Principle of the proposed TSH TR-IFMA represented by the final immunocomplex of the sandwich assay. SA: Streptavidin. α, β: α and β subunit of TSH molecule.

EXPERIMENTAL

Chemicals and Buffers

Affinity-purified Fc-specific goat anti-mouse IgG (Code: G5-Mg16) was purchased from OEM Concepts Inc. (Toms River, New Jersey). Six clones of affinity-purified anti-hTSH McAbs were obtained from different sources; McAb2 was from XieHe Hospital (Beijing, China); McAb1, McAb3, McAb4, and McAb5 (Clone No: 5401, 5403, 5404, and 5405, respectively) were from Medix Biochemica (Helsinki, Finland), and McAb6 was from our department. Sheep anti-hTSH polyclonal anti-serum was from our department and purified with a two-step precipitation procedure as described by Reik and co-workers.(20) Microtitration strips were products of NUNC Co. (Denmark). *N*′-[*p*-Isothiocyanato-benzyl]-diethylene-triamine-N^1,N^2,N^3,N^3-tetraacetate-Eu^{3+} (DTTA-Eu^{3+}) was from Tianjing Radio-Medical Institute (Tianjing, China). BSA was from Shenzhen JingMei Bioengineer Co. (Shenzhen, China). Streptavidin, Biotinamidocaproate *N*-Hydroxysuccinimide ester (BAC-NHS) and the highly purified hTSH were products of Sigma Chemical Co. (St. Louis, MO, USA). TSH calibrators were prepared by diluting the concentrated TSH stock solution to the desired concentrations with neonatal calf serum. The enhancement solution for Eu^{3+} dissociation and fluorescence enhancement was prepared according to Hemmla et al. (21) generally with a fluorescence background of less than 550 CPS. Other reagents were purchased from Sigma Chemical Co.

The coating buffer was 100 mmol/L sodium carbonate buffer (pH 9.0), containing 0.9% NaCl and 0.04% NaN_3. The blocking buffer was 50 mmol/L *Tris*-HCl, pH 8.0, containing 0.9% NaCl, 0.04% NaN_3, and 1% BSA. Assay buffer A used for dilution of the biotinylated antibodies was 50 mmol/L *Tris*-HCl, pH 7.8, containing 2% BSA, 0.04% NaN_3, 0.9% NaCl, 0.1% Tween-20, 0.05% bovine globulin, and 0.5% normal mouse serum. Assay buffer B, used for dilution of the europium labeled streptavidin, was similar to assay buffer A except that mouse serum was omitted and Tween-20 was reduced to 0.04%. The wash solution was double-distilled water containing 0.04% Tween-20 and 0.9% NaCl, with the pH adjusted to 8.4 by *Tris*. TSA buffer for elution of the europium labeled reagents was 50 mmol/L *Tris*-HCl, pH 7.8, containing 0.9% NaCl and 0.05% NaN_3.

Human Serum Samples

54 samples from hyperthyroid patients and 51 samples from euthyroid patients, together with the hTSH values measured by ACS-180 CLIA, were

provided by 301 Hospital (Beijing, China). All the patients have been diagnosed on the basis of characteristic clinical features and confirmed by laboratory tests. The detailed pathologic situations of these patients were not given.

Instrumentation

The chromatographic separation system was a product of Bio-Rad Co., including Model EP-1 Econo Pump and Model EM-1 Econo UV monitor. The fluorescence was measured by an Arcus 1230 fluorometer (LKB-Wallac, Finland) with the excitation and emission wavelength defined at 340 nm and 613 nm, respectively. The 1296-003 DELFIA plateshake and 1296-026 DELFIA platewash were products of Perkin Elmer-Wallac. Manual pipetting was done with disposable plastic tips and Finnpipette (Labsystem Oy, Helsinki, Finland). The 1260 Multigamma II Gamma Counter was a product of LKB-Wallac. Centricon-30 (30-kDa cut-off) micro-concentrator was a product of Poll-Gelman Co.

Microwell Coating

Concentrated McAb5 (1 mg/mL) was diluted to 5 mg/mL in coating buffer. 200 μL of the diluted antibodies was added per well and incubated overnight at room temperature (RT). The strips were then aspirated and washed twice with wash solution; 300 μL of blocking buffer was dispensed into each well and the strips were incubated for 4 h at RT. After decanting the solution in the wells, the strips were stored at 4–8°C in a sealed moist bag. Coating the sheep anti-TSH polyclonal antibodies was done in the same way.

Europium Labeling of Streptavidin, Goat Anti-Mouse IgG and Anti-hTSH McAbs

1 mg of lyophilized streptavidin was dissolved in 50 mmol/L sodium carbonate buffer (pH 9.8), containing 0.9% NaCl. This solution was added to a glass vial containing 0.8 mg of DTTA-Eu^{3+}. Immediately after this, the mixture was agitated for 1 min and then incubated overnight at RT. The labeled streptavidin was separated from excess free label by gel filtration using a Sepharose CL-6B column (1.5×50 cm) and TSA elution buffer. The elution was monitored at 280 nm with a Model EM-1 Econo UV monitor, and a total of 8.8 mL of labeled streptavidin was collected. The concentration

of streptavidin in the purified conjugate solution was determined with the Beer-Lambert method (the UV absorbed by the DTTA-Eu was subtracted); Streptavidin at 1 mg/mL gave an absorbance of 19 at 280 nm. The Eu^{3+} concentration was measured against Eu^{3+} calibrators in the 1230 fluorometer after fluorescence enhancement. The average number of Eu^{3+} coupled to one streptavidin was 9.4, calculated from the molar concentrations of Eu^{3+} and streptavidin. The labeling of goat anti-mouse IgG and anti-hTSH McAbs (McAb3 and McAb4) was performed identically to the procedure for streptavidin.

To test the influence of labeling extent of streptavidin on both of the specific fluorescence response and the non-specific background, a fixed amount of streptavidin (0.2 mg) was labeled with 5–250-fold molar excess of the labeling agent (0.011–0.55 mg). After purification and determination of the Eu^{3+}-to-protein ratios, the utility of the different conjugates was evaluated as follows: one strip of wells (12 wells per strip) coated with McAb5 was washed twice with wash solution; to each well, duplicate 100 μL of calibrators with 20 mIU/L of hTSH were added, followed by addition of 100 μL of assay buffer A containing 100 ng of biotinylated McAb3 (see *Biotinylation of anti-hTSH McAbs* section). The mixture was incubated for 4 h at RT with slow shaking. The wells were washed two times, and 200 μL of assay buffer B containing 50 ng of Eu^{3+}-streptavidin labeled at different extents was added. The strip was incubated for 30 min at RT with shaking. The wells were washed six times, 200 μL of enhancement solution was added to dissociate the bound Eu^{3+} and enhance its fluorescence after 8 min of development. The effect of the labeling extent of streptavidin on the background was investigated in a similar way, but the calibrator with 20 mIU/L of hTSH was replaced by neonatal calf serum that was used as zero calibrator in our assay.

Selection of the Three Mutually Paired Monoclonal Anti-TSH Antibodies

To choose three McAbs that can form a suitable sandwich pair between each other, we modified the method previously reported by Kwak and Yoon (22) and tested six clones of anti-hTSH McAbs: 100 μL of hTSH calibrator, at a concentration of 40 mIU/L, was added to the 12 microwells of the one strip that had been coated with sheep anti-hTSH IgG. 300 ng of anti-hTSH McAb1 (the first one of the six McAbs to be evaluated) in 100 μL of assay buffer A was added. The mixture was incubated for 8 h at RT with slow shaking, to allow hTSH to react with the anti-hTSH McAb1 to saturation. The strip was washed twice with wash solution, 200 μL of assay buffer B containing 100 ng of McAb1 was added to the first two wells

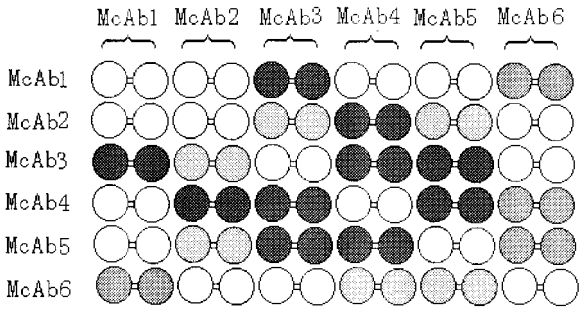

Figure 2. The pairing information of the six anti-hTSH McAbs studied. The depth of the color corresponds to the fluorescence intensity after combining each possible antibody pair. (open circles): Fluorescence below 5000 CPS; (light grey circles): fluorescence between 100 000 and 200 000 CPS; (dark grey circles): fluorescence greater than 200 000 CPS.

to serve as control, then duplicate 100 ng of each of the other five McAbs in 200 μL assay buffer B was added successively into the left 10 wells. The strip was incubated for 4 h. After twice aspirating and washing the strip, 200 μL of assay buffer B containing 100 ng of europium labeled goat anti-mouse IgG was added to each well and incubated for 2 h at RT with shaking.

After this, the strips were washed six times, 200 μL of enhancement solution were added to dissociate the europium ion from the surface immuno-complex into solution where they formed highly fluorescent chelates with components in the enhancement solution. The fluorescence was measured with the Model 1230 time-resolved fluorometer. The pairing information of McAb1 with each of the five McAbs can thus be obtained, where an increased fluorescence reading compared with the control indicated that the two antibodies have bound to different epitopes of the TSH molecule.

The same procedure was applied to evaluate the pairing information of the left five McAbs with each of the other McAbs. McAb 3, 4, and 5 were finally selected for our TR-IFMA because the combination of the three antibodies was able to generate the highest fluorescent response, as shown in Figure 2.

Biotinylation of Anti-hTSH McAbs

Of the three selected anti-hTSH McAbs, the McAb3 (specific to the intact TSH molecule) and McAb4 (mainly directed to the β-subunit of TSH)

were biotinylated and used as detection antibodies because of their relatively lower nonspecific binding characteristic compared to McAb5 (mainly directed to the β-subunit of TSH). Before biotinylation, the mixture of the two antibodies, at a concentration of 0.5 mg/mL each, was first dialyzed against 1000 mL of 0.1 mol/L sodium carbonate buffer (pH 9.4) for 48 h in tubing with Mr-2000 cutoff, then transferred to a small, clean glass bottle. 40 μL of BAC-NHS solution, which was freshly prepared before use by dissolving 10 mg of BAC-NHS in 2 mL of *N*,*N*-dimethylformamide, was added to the antibody solution. The contents was promptly mixed, covered with Parafilm, and incubated for 90 min at ambient temperature. After that, the mixture was transferred to dialysis tubing and dialyzed against 4000 mL of 0.1 mol/L *Tris*-HCl buffer (pH 8.0), containing 0.9% NaCl and 0.05% NaN_3. The dialysis incubation was allowed to proceed for two days. The biotinylated antibodies were filtered through a 0.2 μm filter and stored at 2–8°C.

The effect of biotinylation extent of the McAbs on both the specific and non-specific fluorescence was also tested. Various amounts of BAC-NHS were used to biotinylate 200 μg of antibody mixture (100 μg of each of McAb3 and McAb4); the molar ratios of BAC-NHS to immunoglobulin were tested in a range from 1 to 500. The biotinylation procedure was similar to that described above, except that the exchange of the antibody matrix and the removal of the free biotin were performed by ultrafiltration using Centricon-30 instead of by dialysis, due to the low volume of the antibody solution.

hTSH TR-IFMA

Microwells coated with anti-hTSH McAb5 (β-subunit specific) were washed twice with wash solution. Duplicate 100 μL of calibrators or serum samples and 100 μL of assay buffer A containing 100 ng of each of the biotinylated McAb3 and McAb4 were added successively. The mixture was incubated for 4 h at RT with continuous slow shaking. Then, the reagents were aspirated and the wells were washed four times. 200 μL of assay buffer B, containing 50 ng of europium labeled streptavidin, was added and incubated for 30 min at RT. The wells were washed six times, 200 μL of enhancement solution was added, and the strips were agitated slowly for 5 min; the fluorescence was measured for 1 s in a 1230 time-resolved fluorometer. For comparison, two identical procedures were performed with only one of the detection antibodies (biotinylated McAb3 or McAb4, 200 ng/well) included in the assay.

To evaluate the effect of biotin-streptavidin interaction on the signal amplification, another hTSH TR-IFMA procedure, using the europium

labeled McAb3 and McAb4, instead of the biotinylated McAbs, was also carried out. The total amount of the detection McAbs and the incubation time was kept unchanged to the biotinylated antibody based TR-IFMA.

Methods for Comparison

Two commercially available hTSH immunoassays, ACS-180 hTSH CLIA from Corning Co. and hTSH IRMA from our department, were used for a comparison study. hTSH CLIA was performed in 301 Hospital (Beijing, China) according to the instructions enclosed in the kits. Serum hTSH IRMA was performed in our lab as follows: 200 μL of hTSH calibrators or samples were pipetted into the pre-washed tubes containing the immobilized anti-hTSH McAb. 50 μL of ^{125}I-labeled anti-TSH tracer solution was added and the contents of the tubes were incubated overnight at 6°C. The reagents in the tubes were decanted, and the tubes were washed four times with 1 mL of wash solution. The radioactivity in each tube was counted for 1 min in the 1260 Multigamma II Gamma Counter.

RESULTS

Antibody Pairing Study

In the present work, six clones of anti-hTSH McAbs were studied to choose three antibodies that can form suitable sandwich pairs between each random couple. In the proposed protocol, the TSH molecules were first captured by the solid phase polyclonal anti-TSH antibodies and saturated by one McAb that was to be evaluated after a 6-h incubation. Then, a second incubation with the same (for control) and different (for analysis) McAbs was carried out. Finally, a europium labeled goat anti-mouse IgG was used to bind the surface McAbs and, finally, to show their amount after fluorescence development. If the second incubation with the different McAbs didn't cause an obvious fluorescence increase or the increase was totally absent, it was concluded that the two McAbs bind to the same or overlapped epitope(s) of the TSH molecule, and can't be employed to develop a sandwich assay. The fluorescence readings shown in Figure 2 indicated that McAb3, McAb4, and McAb5 were the most favorable combination because they were able to bind to the same TSH molecule simultaneously and exhibited the highest specific fluorescence. Of the three selected McAbs, biotinylated McAb5 showed a more obvious non-specific

binding feature, compared to McAb3 and McAb4 (data not shown). So, McAb3 and McAb4 were used as detection antibodies in our assay.

Optimization of Streptavidin Labeling and Antibody Biotinylation

The labeling extent of streptavidin is an important factor affecting both the background and specific signals and even the storage stability of the conjugates. Figure 3 shows that the specific signals at 20 mIU/L of TSH concentration had approximately a linear correlation with the conjugated Eu^{3+} number per streptavidin until the Eu^{3+}/streptavidin ratio reached approximately 11. The drift away from linearity when the streptavidin contained >11 Eu indicated a negative effect on its binding activity. Also, the higher background which arose from the over-labeling of streptavidin caused a severe decrease on the final signal-to-noise ratio. The Eu/streptavidin was controlled near 10.

The extent of biotinylation of McAbs was also optimized. A very similar result as that for streptavidin labeling was observed, in which excessive or insufficient biotinylation was detrimental to the overall signal-to-noise ratio. The optimal amount of BAC-NHS used to biotinylate 500 μg of IgG was about 0.025 mg.

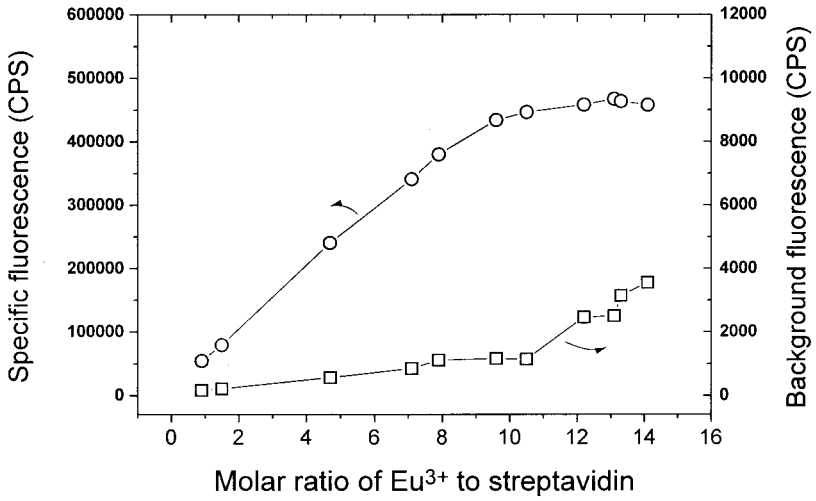

Figure 3. The effect of streptavidin labeling degree on both of the specific (circle) and background (square) fluorescence. The concentration of the calibrator used was 20 mIU/L.

Calibration Curve, Assay Range, Precision, and Sensitivity

A typical calibration curve and precision profile are shown in Figure 4. The linear response extends beyond 100 mIU/L of TSH. The highest fluorescence at 150 mIU/L of TSH was about 8 500 000 CPS. Widening the calibrator range up to 200 mIU/L or more is unsuitable because the fluorescence was too high to be correctly detected. However, if required, the assay range can be easily extended by appropriate dilution of the samples prior to testing. The analytical sensitivity was 0.002 mIU/L, when defined as the background signal +2SD ($n = 12$). A 30-min incubation of the Eu^{3+}-streptavidin with the surface immunocomplex at RT was enough to give >75% of the maximum fluorescence; prolonging this incubation didn't improve the sensitivity further because the background increased more than the specific signal. The functional sensitivity was 0.017 mIU/L when 20% of the inter-assay CV was regarded as the acceptable limit, as shown in the inter-assay precision profile (Figure 5).

Dilution Reliability and Recovery Test

Four clinical samples containing TSH in the range of 0.92 to 137.4 mIU/L were diluted 2–64-fold with neonatal calf serum. The concentrations

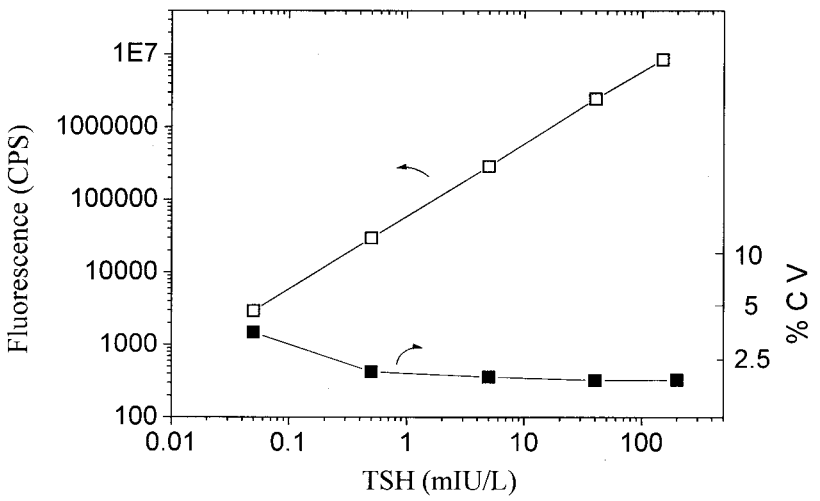

Figure 4. Typical calibration curve and precision profile ($n = 12$) obtained by present hTSH TR-IFMA. The background signals were subtracted.

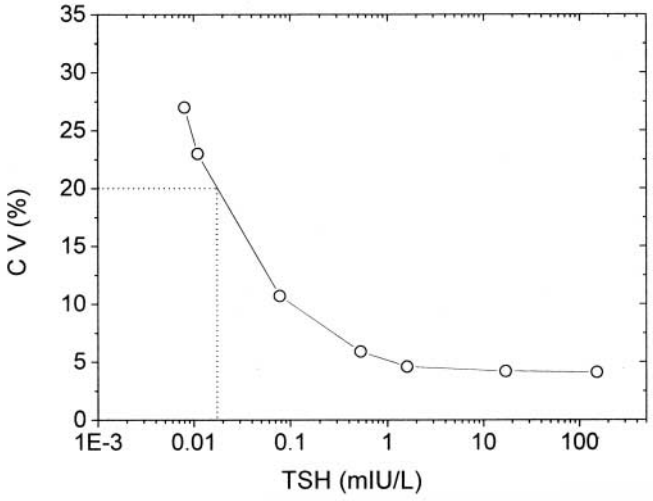

Figure 5. The inter-assay precision profile ($n = 13$) of the present hTSH TR-IFMA.

Table 1. Dilution Test of Present hTSH TR-IFMA

Dilutions	hTSH Measurements (mIU/L)			
None	**137.4**	16.8	**8.58**	**0.92**
1/2	**65.74**	8.4	**4.30**	**0.45**
1/4	**35.56**	4.2	**2.31**	**0.23**
1/8	**17.91**	2.1	**1.15**	**0.12**
1/16	**9.06**	1.1	**0.62**	**0.065**
1/32	**4.37**	0.56	**0.30**	**0.031**
1/64	**2.31**	0.29	**0.15**	**0.013**

determined by the present TR-IFMA are shown in Table 1. There was no significant deviation from the linearity relationship between the measurements and amounts of dilution, even when hTSH concentrations were diluted down to a level approaching the functional sensitivity. Meanwhile, no loss of power was observed for measuring serum at a high TSH concentration (e.g., >100 mIU/L). The analytical recovery was studied by adding a known amount of TSH to three clinical samples with different TSH concentrations; the TSH concentrations were determined before and after the addition by the present method. The recovery shown in Table 2 ranges from 89.7 to 102.6%.

Table 2. Analytical Recovery of hTSH Added to Serum

TSH (mIU/L)			
Original	Added	Observed	% Recovery
0.217	0.029	0.243	89.7
0.920	0.078	1.000	102.6
18.5	2.58	21.0	96.9

Sensitivity Enhancement by Using Double Detection Antibody and Biotin-Streptavidin Amplification

Four calibration curves were obtained by simultaneously using two biotinylated McAbs, using each of the two biotinylated McAbs and using two Eu-labeled McAbs. All factors in the TR-IFMAs were kept constant with only the detection antibody changed. As shown in Figure 6, the signals produced by using two biotinylated McAbs were about 1.5- and 2.5-times of that produced by using individual biotinylated McAb4 or McAb3, respectively. The biotin-streptavidin interaction amplified the fluorescence more than 5-times, compared to that using the europium-directly-labeled McAb3 and McAb4. The background in the four cases was of the same level when the total concentration of the detection antibodies was kept constant.

Cross-Reactions

When the LH and FSH tested were at 200 mIU/L, the apparent TSH concentrations were 0.0406 and 0.0192 mIU/L, respectively. No signal was generated with hCG at 2500 IU/L. Whether these cross-reactions were caused by the trace amount of contaminated hormones was not identified. However, such cross reactivity would not affect the present assay.

Correlation

The correlation of the results obtained by the present method with those obtained by the ACS-180 CLIA was studied by measuring TSH in 89 clinical samples (only euthyroid and hypothyroid subjects included). The data were analyzed using a linear regression plot, as shown in Figure 7. The correlation was excellent over the whole TSH concentration range

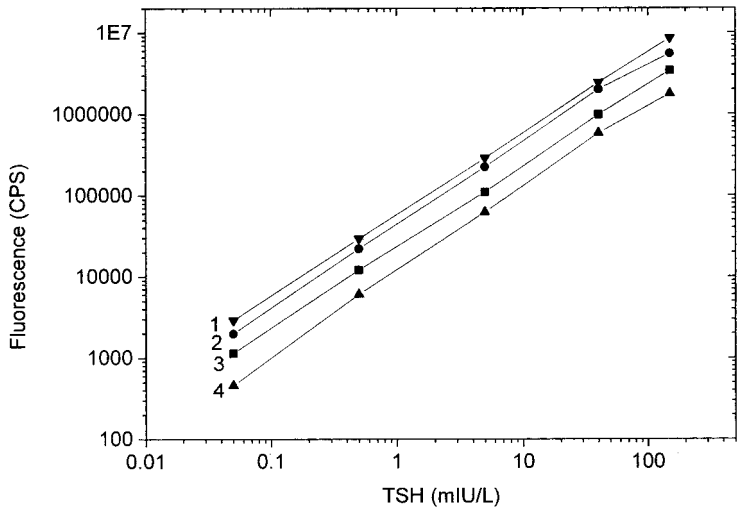

Figure 6. Calibrator curves of the hTSH TR-IFMA based on biotinylated McAb3 and McAb4 (curve 1), biotinylated McAb4 (curve 2), biotinylated McAb3 (curve 3), and europium directly labeled McAb3 and McAb4 (curve 4). The total amount of the detection antibodies was kept constant.

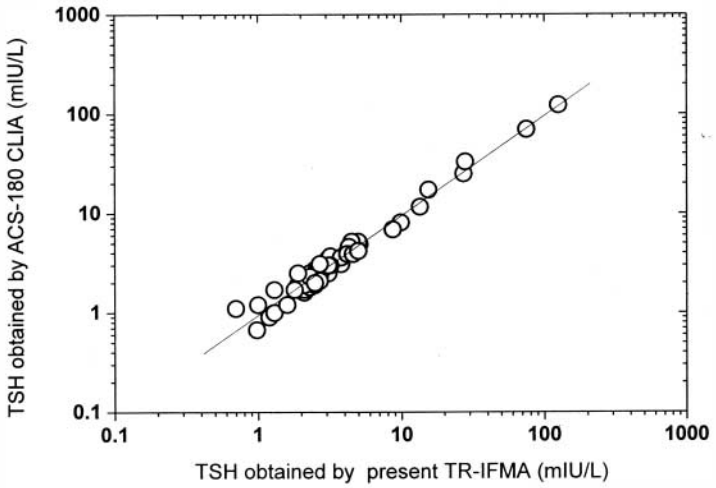

Figure 7. Correlation between the values obtained by present TR-IFMA and that obtained by ACS-180 CLIA. Only euthyroid and hypothyroid samples were analyzed ($n = 89$).

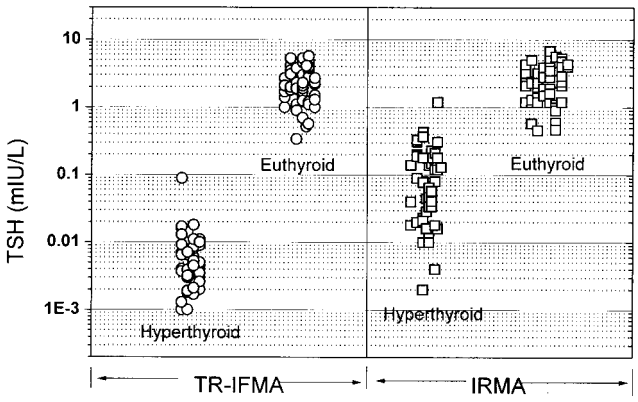

Figure 8. Distribution of the serum hTSH concentrations in hyperthyroid and euthyroid groups determined by present TR-IFMA (circle) and IRMA (square). 49 samples from hyperthyroid patients and 51 samples from euthyroid patients were analyzed.

($R = 0.9855$). The slope was 1.003 and the y-intercept was insignificant (-0.028).

Analysis of Serum from Hyperthyroid and Euthyroid Individuals

We measured 54 samples from hyperthyroid patients and 51 samples from euthyroid patients with the present TR-IFMA and IRMA (Figure 8). The correlation between the two methods was good ($r = 0.967$) when only the values of euthyroid subjects were evaluated. However, the TR-IFMA showed much improved performance for low TSH concentration. Of the 54 hyperthyroid samples, 53 samples measured by TR-IFMA gave TSH values <0.02 mIU/L; TSH concentrations in 5 samples remained undetectable (fluorescence reading slightly below that of the zero calibrators); and only one sample had a TSH value approaching 0.1 mIU/L. With IRMA, 20 samples had TSH values >0.1 mIU/L, ranging from 0.12 to 0.9 mIU/L, causing some extent of overlapping with the measurements of euthyroid subjects.

DISCUSSION

(Strept)avidin-biotin interaction has been extensively documented as a versatile mediator for various immunoassay configurations.(17,18,23)

However, the reports of immunoassays that simultaneously employ two or more detection antibodies to improve the sensitivity are fairly scarce. An early paper described an EIA for carcinoembryonic antigen (CEA), where three McAbs recognizing different epitopes of CEA were used,(24) but differently, two of the McAbs were used as capture antibodies instead of being used as detection antibodies. The commercial Wallac hTSH DELFIA Ultra adopted a double detection antibody.(25) However, the Eu^{3+} was used directly to label the detection antibodies and no article is available describing the developmental details. To our knowledge, this is the first report of a TR-IFMA that combines both a double detection-antibody strategy and streptavidin-biotin amplification.

DELFIA technology was employed in this assay. McAb5, which exhibited a relatively high non-specific binding, was used to coat the microwells, and McAb3 and McAb4 were biotinylated as detection antibody. An analytical sensitivity of 0.002 mIU/L and a functional sensitivity of 0.017 mIU/L were achieved, which is comparable to that of the most sensitive TSH immunoassays ever reported.(26) The highly sensitive detection of the present assay can be attributed to several factors that were simultaneously involved in our development. Firstly, the high detectability of the Eu^{3+} label in the fluorescence enhancement solution ($\sim 10^{-14}$ mol/L) and the satisfactory labeling behavior of the bi-functional chelate (DTTA-Eu^{3+}) make the DELFIA an intrinsically very sensitive technology. Secondly, the streptavidin-biotin interaction, together with the double detection antibody binding, amplified the specific signals to more than 10 times of that when no such strategies were taken. Thirdly, the labeling extent of the streptavidin, the biotinylation extent of the McAbs, and the components of the assay system were well optimized to suppress the background to a low level, so the signal-to-noise ratio was further improved.

Choosing suitable antibody pairs is an essential step for developing sensitive McAb-based sandwich immunoassays. In most cases, two McAbs (one as capture antibody and another as detection antibody) are used to form a typical sandwich after binding to their target antigen. However, when a higher sensitivity is desirable, it can be expected that employing two or more detection antibodies will favor increasing the specific response and, subsequently, the analytical sensitivity. The McAbs useable for this purpose must not be mutually exclusive between each random couple in binding the analyte. The proposed protocol for determining if two McAbs can be paired to develop a sandwich assay is convenient and efficient. This procedure requires only one surface binder (namely, sheep anti-hTSH polyclonal antibodies in this work) and one labeled second antibody, both of which can be commonly applied to screen all of the anti-hTSH McAbs. Thus, the tedious work for labeling individual antibodies and preparing

different surface antibodies which were included in the routine method are circumvented. Additionally, the inconsistency in labeling and coating individual antibodies is eliminated; so, the fluorescence increment caused by the binding of other antibodies provides more reliable information of the compatibility of the two McAbs in binding their antigen.

Streptavidin is particularly suitable to be used as a label carrier because of its stable, low specificity binding properties; high affinity; and four binding sites for biotin. Also, the bi-functional chelate DTTA-Eu^{3+} used in our assay has been widely accepted as a mildly hydrophilic and an efficient coupling agent that does not exert much adverse effect on the labeled reagent. However, when streptavidin was extensively conjugated with Eu^{3+}, the binding property of streptavidin to biotin was weakened to some extent, and an increased non-specific signal was observed (Figure 3). Moreover, some aggregates always resulted from over-labeling, which not only lowered the yield of the desired conjugates, but also deteriorated storage stability of the labeled reagent, even after the aggregates were separated (data not shown). Because the $-NH_2$ in streptavidin, available for conjugating with DTTA-Eu^{3+}, is not directly involved in its binding site, the above phenomenon suggests that the charge or/and the configuration of streptavidin may be distorted by over-labeling and its binding for biotin may, thus, be affected sterically. Similar results were observed for the biotinylation of McAb3 and McAb4. Extensive biotinylation of the McAbs in the presence of a large excess amount of BAC-NHS also gave rise to suppressed specific binding and an increased background. This, perhaps, was caused by blocking or deforming of the combining sites of the antibody after the introduction of the biotin moiety and then the antibody's reaction with the TSH molecule was inhibited. The increased hydrophobicity caused by the attached biotin may also have contributed to the elevated background. To our experience, the biotinylation extent that an antibody can tolerate differs from antibody to antibody and should be determined empirically.

The aim of this work was to use double detection antibodies and biotin-streptavidin interaction to enhance the sensitivity of the hTSH TR-IFMA, and to further improve its diagnostic value in separating hyperthyroid from euthyroid conditions. As shown in Figure 8, hTSH values of hyperthyroid subjects measured by the present TR-IFMA fall clearly below the normal range while overlaps of TSH values exist for IRMA. Although such a result is not enough to predict that our method can offer a complete cut between the two clinical situations, due to the relatively small number of samples tested, it is clear that the present TR-IFMA allows a more efficient discrimination between hyperthyroid and euthyroid hTSH values. The inability of the present method to detect hTSH from the

five hyperthyroid samples may be due to the inconsistency of the matrix between the calibrators and the serum samples. Such a matrix difference may influence both the immunoreaction rate and the extent of non-specific binding and, subsequently, result in an undetectable value in case the hTSH concentration is extremely low (near zero). In the future, it is still interesting to use other strategies, such as ligand-label based TRFIA, *acidic* co-fluorescence enhancement, multiple labeling, etc., to further improve the sensitivity of immunoassays. These works will certainly be of great assistance for many purposes (e.g., for detection of the trace amounts of p24 antigen in AIDS screening) and are presently in progress in our laboratory.

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