

Ultrasensitive Thyrotropin Immunoassay Based on Enzymatically Amplified Time-Resolved Fluorescence with a Terbium Chelate

Anastasia Papanastasiou-Diamandi,¹ Theodore K. Christopoulos,² and Eleftherios P. Diamandis²⁻⁴

We describe an ultrasensitive, enzymatically amplified time-resolved fluorescence immunoassay of thyrotropin (thyroid-stimulating hormone) in serum with use of a terbium chelate as the detectable moiety. In this assay, thyrotropin is first simultaneously reacted with a solid-phase (microtiter well) monoclonal antibody and a soluble biotinylated monoclonal detection antibody. After washing, a streptavidin-alkaline phosphatase conjugate is added, followed by another washing. Alkaline phosphatase acts on the substrate 5-fluorosalicyl phosphate (FSAP) to produce 5-fluorosalicyl acid (FSA). FSA, but not FSAP, can then form with Tb³⁺ and EDTA a highly fluorescent ternary complex of long fluorescence lifetime. This complex is quantified with time-resolved fluorometry. The thyrotropin assay is highly sensitive (detection limit ~0.003 milli-int. unit/L when a total assay time of 85 min is used), precise, and accurate. The thyrotropin assay can also be completed in <30 min (detection limit 0.013 milli-int. unit/L), thus making this procedure a candidate technology for high-throughput automated analyzers.

Additional Keyphrases: *nonisotopic immunoassay · lanthanide chelates · biotin-streptavidin interaction*

There are many highly sensitive nonisotopic thyrotropin (TSH) immunoassays based on fluorescent, luminescent, or enzymatic labeling systems (1-7).⁵ Recently, enzymatic amplification has been combined with chemiluminescence detection. These systems are exceptionally sensitive and are finding applications in immunoassays and nucleic acid hybridization assays (8, 9). Highly sensitive assays have also been reported for TSH and other analytes, in which europium chelates labels and time-resolved fluorometry are used (10, 11). Recent efforts by our group and others have been directed toward combining enzymatic amplification and time-resolved fluorometry to devise new highly sensitive detection methodologies. One such combination, based on the work of Bobrow et al. (12, 13), was already reported (14).

Recently, Evangelista et al. (15) reported the synthesis of various enzyme substrates [including alkaline phosphatase (ALP) substrates] that are derivatives of 5-fluorosalicyl acid (FSA). The unique property of these substrates is that they cannot form ternary fluorescent complexes with Tb³⁺ and EDTA in their native form but do so when an enzyme cleaves off a masking group on the hydroxyl group of FSA. The ternary fluorescent complexes emit fluorescence that is long-lived and is characteristic of Tb³⁺, in accordance with the energy-transfer principles described in detail in a recent review (16).

Here we describe the development of an ultrasensitive immunoassay of TSH, based on the principles of enzymatic amplification, the biotin-streptavidin system (17), and time-resolved fluorometry with Tb³⁺ chelates. This assay is highly sensitive, precise, and accurate and can be completed in <90 min. Other experiments showed that high sensitivity can be maintained even with a total incubation time of <30 min, thus making the technology an attractive candidate for fully automated high-throughput immunoassay analyzers.

Materials and Methods

Materials and solutions. The phosphate ester of 5-fluorosalicyl acid (FSAP) was obtained from CyberFluor Inc. (Toronto, Canada) either as a solid reagent (4.7 mg/vial) stored at -20 °C and reconstituted in 2 mL of NaOH (0.1 mol/L) or as a 0.01 mol/L solution in NaOH (0.1 mol/L) stored at 4 °C. ALP-labeled streptavidin (SA-ALP) was obtained from Zymed Labs. Inc. (San Francisco, CA) as a 0.75 g/L stock solution. Working SA-ALP solution was prepared by diluting the stock solution 5000-fold in the bovine serum albumin (BSA; 60 g/L) diluent described below. White, opaque, 12-well polystyrene microtiter strips coated with a monoclonal anti-TSH antibody were obtained from CyberFluor. The stock biotinylated detection antibody (60 mg/L) and TSH assay buffer were also available from CyberFluor and are described elsewhere (11). The working biotinylated antibody solution was prepared by diluting the stock solution 50-fold in the assay buffer. The substrate buffer is a Tris buffer (0.1 mol/L, pH 9.0) containing 0.1 mol of NaCl and 1 mmol of MgCl₂ per liter. The substrate working solution (FSAP, 1 mmol/L, in substrate buffer) was prepared just before use by diluting the FSAP stock solution 10-fold in substrate buffer. The SA-ALP diluent was a 60 g/L solution of BSA in 50 mmol/L Tris buffer, pH 7.40, containing 0.5 g of sodium azide per liter. The wash solution was prepared by dissolving 9 g of NaCl and 0.5 mol of polyoxyethylenesorbitan monolaurate (Tween 20) in 1 L of distilled water.

¹ CyberFluor Inc., 179 John St., Toronto, Ontario, Canada M5T 1X4.

² Department of Clinical Biochemistry, Toronto Western Hospital, 399 Bathurst St., Toronto, Ontario, Canada M5T 2S8.

³ Department of Clinical Biochemistry, University of Toronto, 100 College St., Toronto, Ontario, Canada M5G 1L5.

⁴ To whom correspondence should be addressed, at the Toronto Western Hospital.

⁵ Nonstandard abbreviations: TSH, thyrotropin (thyroid-stimulating hormone); FSA, 5-fluorosalicyl acid; FSAP, 5-fluorosalicyl phosphate; SA, streptavidin; ALP, alkaline phosphatase; and BSA, bovine serum albumin.

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TSH standards, 0–100 milli-int. units/L in TSH-free human serum matrix, were obtained from CyberFluor.

The developing reagent is a Tb^{3+} -EDTA solution in Tris buffer and is prepared by mixing one volume of 5 mmol/L Tb^{3+} -EDTA solution in HCl (0.01 mol/L), one volume of 2.5 mol/L Tris buffer (pH 13.0), and three volumes of water. The final composition of this solution is Tb^{3+} -EDTA complex, 1 mmol/L, and Tris, 0.5 mol/L.

Instrumentation. For measuring liquid-phase Tb^{3+} fluorescence in white microtiter wells, we used the CyberFluor 615™ Immunoanalyzer, a time-resolved fluorometer. The time-gate settings of the instrument were not changed from those described elsewhere (18) because the fluorescence lifetimes of the ternary complexes of Tb^{3+} with EDTA and salicylate derivatives are similar to those of the fluorescent Eu^{3+} complexes. The same interference filter of the emission pathway was also used (615 nm).

Assay procedure. Pipet 100 μ L of the standard or samples into each coated microtiter well and add 50 μ L of the working biotinylated antibody solution. Incubate with mechanical shaking for 60 min at room temperature and then wash four times. Add 100 μ L of the SA-ALP phosphatase working solution to each well, incubate 15 min as above, and wash four times. Add 100 μ L of the 1 mmol/L working FSAP substrate solution to each well and incubate for 10 min as above. Then add 100 μ L of the developing reagent per well, mix by mechanical shaking for 1 min, and measure the fluorescence with the time-resolved fluorometer. The calibration curve and data reduction are carried out automatically by the analyzer.

Results and Discussion

The ternary fluorescent complex of Tb^{3+} , EDTA, and FSA has an excitation wavelength of 336 nm (characteristic of FSA) and emission maxima at 491, 548, 587, and 624 nm (characteristic of Tb^{3+}). The complex exhibits maximum fluorescence intensity at pH 13.0. Tb^{3+} fluorescence was measured by using the unmodified 615 Immunoanalyzer (18), which has a nitrogen laser exciting at 337.1 nm and an interference filter in the emission pathway with a nominal wavelength of 615 nm, the emission maximum of Eu^{3+} . We used the same instrument to quantify Tb^{3+} fluorescence at 615 nm, which is close to the Tb^{3+} peak at 624 nm. Although this peak is much smaller than the Tb^{3+} peak at 548 nm, we found no benefit in measuring Tb^{3+} at any other characteristic wavelength because of the parallel increase in the background signal obtained with the zero standard. Overall, the signal-to-background ratio was similar at all five wavelengths tested (four peak wavelengths and 615 nm).

The TSH assay was optimized with respect to dilution of detection antibody, streptavidin dilution, and incubation times. In all cases, we selected conditions that maximize the ratio of signal between the 0.05 milli-int. unit/L standard and the zero standard. Results are shown in Figure 1. From these data we determined the following optimal conditions: biotinylated antibody, 60

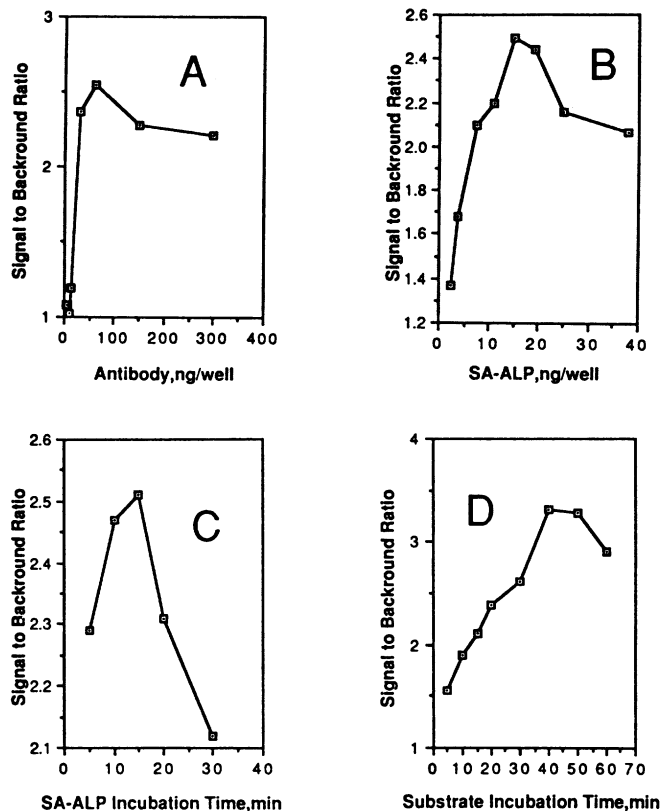


Fig. 1. Optimization of the proposed TSH assay

The signal-to-background ratio is the ratio of fluorescence obtained with the 0.05 and 0 milli-int. unit/L standards. A: optimization of antibody amount; B: optimization of the amount of SA-ALP conjugate; C: optimization of the SA-ALP incubation time; D: optimization of the substrate incubation time. In all cases, the first incubation was for 1 h

ng in 50 μ L/well; SA-ALP conjugate, 15 ng in 100 μ L/well; and SA-ALP incubation time, 15 min. Although the substrate incubation time of 40 min gave the best signal-to-noise ratio, this was too long and the dynamic range of the assay was limited. Incubation times were selected as follows: 60 min for the first step,

Table 1. Precision of the Proposed TSH Assay^a

TSH, milli-int. units/L		
Mean	SD	CV, %
Within-run (n = 24)		
0.110	0.0057	5.2
0.220	0.017	7.9
0.56	0.016	2.8
1.37	0.049	3.6
6.63	0.17	2.6
12.81	0.74	5.8
Day-to-day (n = 12)		
0.230	0.0145	6.3
0.360	0.022	6.0
1.25	0.060	4.8
2.28	0.090	4.2
5.07	0.20	4.0
7.86	0.32	4.0

^a Protocol with 85-min total incubation.

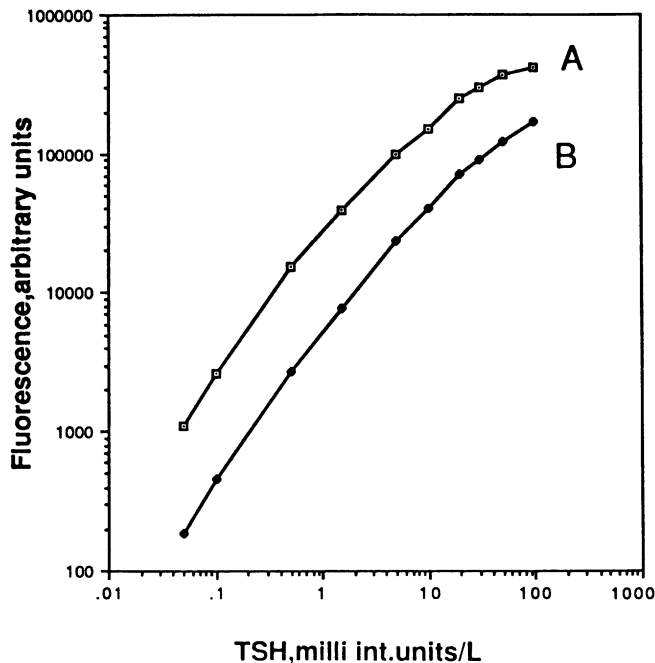


Fig. 2. Calibration curves obtained with a 60/15/10-min (a/b/c) assay protocol (A) and a 10/5/10-min assay protocol (B)

(a) Incubation with sample and biotinylated antibody; (b) incubation with SA-ALP conjugate, and (c) incubation with the enzyme substrate FSAP

15 min for the second step, and 10 min for the third step. On another occasion, we varied the incubation times to achieve a very quick protocol (<30 min); the results are presented below.

Calibration curves of the developed TSH assay with the 85-min total incubation time protocol and with another protocol of 25-min total incubation time (10, 5, and 10 min, respectively, for the three incubations) are shown in Figure 2. The detection limit, defined as the concentration of TSH corresponding to the fluorescence of the zero standard plus 2 SD of the zero-standard fluorescence ($n = 24$), was 0.003 milli-int. unit/L for the 85-min protocol and 0.013 milli-int. unit/L for the 25-min protocol. Both assays are useful for TSH concentrations up to 50 milli-int. units/L. Within-run precision was assessed by analyzing six samples containing various TSH concentrations ($n = 24$). The within-run CVs ranged from 2.6% to 7.9% (Table 1). Another six samples were analyzed 12 times over a period of two weeks (calibration curve and samples run in duplicate each day) to give day-to-day CVs ranging from 6.3% to 4.0% (Table 1).

Analytical recovery data are shown in Table 2. Recovery ranged from 89% to 97% with a mean of 92% (SD 2.6%).

We analyzed all serum samples by the proposed method and by a commercially available high-sensitivity TSH assay (FIAGEN™; CyberFluor). Sixty-six samples had TSH values <0.1 milli-int. unit/L by both methods. The comparison of the remaining samples was done by linear-regression analysis at two concentration ranges. For 38 samples with TSH values ≤ 12 milli-int. units/L, the regression equation found was y (proposed

Table 2. Analytical Recovery with the Proposed TSH Assay^a

TSH, milli-int. units/L			
Initially present	Added	Recovered	Recovery, %
2.16	8.30	7.85	95
	16.60	15.17	91
4.89	8.30	8.09	97
	16.60	15.51	93
3.12	8.30	7.60	92
	16.60	14.97	90
1.86	8.30	7.65	92
	16.60	14.83	89
1.75	8.30	7.42	89
	16.60	15.02	91
2.23	8.30	7.90	95
	16.60	14.88	90
		Mean	92.0
		SD	2.6

^a Protocol with 85-min total incubation.

method) = $0.998x$ (comparison method) - 0.23 ($r = 0.97$; $S_{y|x} = 0.669$). For 45 samples with TSH values ≤ 50 milli-int. units/L, the regression equation was $y = 1.17x - 1.01$ ($r = 0.99$; $S_{y|x} = 1.25$). The proposed procedure gives slightly higher values at TSH concentrations >25 milli-int. units/L.

Many high-sensitivity TSH assays are based on radioisotopes, time-resolved fluorometry with europium chelates as labels, and enzymes combined with fluorogenic or chemiluminogenic substrates. Most of these assays need prolonged incubation time (>1 h), and are unsuitable for incorporation into fully automated high-throughput systems. In the proposed methodology, the advantages of time-resolved fluorometry (16, 18), of the streptavidin-biotin system (17), and of enzymatic amplification are combined to yield a highly sensitive TSH method (detection limit 0.003 milli-int. unit/L). The method is still highly sensitive (detection limit <0.02 milli-int. unit/L) even if the first incubation step between solid-phase antibody, antigen, and biotinylated detection antibody is only 10 min and the total incubation time is 25 min. We anticipate that this detection methodology will be suitable for use in high-throughput automated immunoanalyzers because the total measurement time per sample is only 1 s, comparing favorably with the measurement times of other competing technologies.

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