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Original Article

Fluorescence immunochip assay for thyroid stimulating hormone in whole blood

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Abstract The determination of blood level of thyroid stimulating hormone (TSH) is an important biomarker for the clinical assessment of thyroid status. Here, we presented a new fluorescence (FL) immunochip TSH assay system, which was developed with a platform of point-of-care test (POCT) for clinical applications. The assay system adopted a lateral-flow immunochromatographic technology and consisted of anti-TSH-mAb coated strip in a disposable chip, a detection buffer containing FL-labeled anti-TSH-pAb, a calibration chip, and a laser FL scanner. The analytical performance of FL immunochip TSH assay system was evaluated by linearity, interference, recovery, and imprecision tests. The comparability of the developed assay was examined with an automated reference assay. The developed assay system exhibited an excellent linearity in working range of 1-100 μIU/mL and was affected neither a large amount of various serum interference substances nor similar structure biomolecules to TSH. The analytical mean recovery of control was 97.6% in a dynamic working range and the imprecision of intra- and inter-assay of CVs was less than 10%. There was a significant correlation between the developed TSH assay and the Beckman Coulter Access 2 TSH assay (r=0.989, $p < 0.001$). The developed FL immunochip assay is the only method that quantifies TSH concentration in whole blood and meets the criteria of POCT including affordable cost, a disposable device, and requiring minimum maintenance to perform test.

Keywords: Anti-TSH-Ab, Fluorescence, Hypothyroidism, Immunoassay, TSH, Point-of-care-testing

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Introduction

Human thyroid stimulating hormone (TSH) is a glycoprotein hormone with a molecular weight of approximately 28 kDa, consisting of two non-covalently bound subunits, α- and β-chain[1,2]. Alpha-chain is almost identical in all species but β-chain is responsible for immunological and biological specificity[3].

TSH is secreted by the anterior lobe of the pituitary gland, and stimulates the production and release of thyroid hormones, thyroxine (T4) and triiodothyronine (T3) by the thyroid gland, which are essential for body metabolism and neural activity[4]. In normal individuals, the levels of these thyroid hormones in blood are maintained in hemostatic equilibrium by a control mechanism on the pituitary. The synthesis and secretion of TSH is regulated by thyrotropin-releasing hormone (TRH) produced in the hypothalamus. Blood levels of TSH and TRH are inversely related to those of the thyroid hormones. When there is a high level of thyroid hormones in blood, less TRH is released by the hypothalamus, so that less TSH is secreted by the anterior pituitary gland. The opposite action will occur when there are decreased levels of thyroid hormones in blood. This process, known as a negative feedback mechanism, is responsible for maintaining the proper blood levels of thyroid hormones[5-7].

The principal clinical use for the determination of

blood level of TSH is for the assessment of thyroid status[8,9]. In patients with intact hypothalamic-pituitary function, TSH is measured to: 1) exclude hypothyroidism or hyperthyroidism; 2) monitor T4 replacement treatment in primary hypothyroidism or anti-thyroid treatment in hyperthyroidism; 3) assess the response to TRH stimulation testing. Serum TSH level is raised in cases of primary hypothyroidism. The diagnosis of hypothyroidism is made by determining a low T4 (T3) value and is confirmed by an elevated TSH level. In hyperthyroidism, levels of T3 and T4 are raised and TSH level is reduced.

The assay methods for serum TSH measurement have been evolved continuously since immunometric assay based on overuse of antibody (Ab) was introduced in the middle of 1980. Currently, assay methods employing new labeling materials, such as, immunoenzymetric assay, immunofluorometric assay, immunochemiluminometric assay, and immunobioluminometric assay, are being popularly used for TSH measurement[10-12]. These assay methods have some advantages in terms of automatic operation and accuracy with high sensitivity and specificity. However, there are still inconvenient in that they require time-consuming procedures, relatively expensive equipment, and a qualified expert to administer.

In this study, we introduced a new TSH assay system with whole blood sample that provided results in 15 min, had a point-of-care-testing (POCT) platform, and had a reliable analytical performance compared to automated reference assay method. This newly developed TSH assay system was based on a traditional lateral flow immunochromatographic technology[13], added two-site sandwich-type Ab scheme, and employed fluorescence (FL) dye as a tracer to detect the level of TSH in samples. The FL immunochip TSH assay system was characterized in details and its functional performances were presented with recovery, imprecision, and interference test. Finally, the clinical application of the new assay method was tested by comparisons of TSH level to well-known reference assay method.

Results and Discussion

Characterization of FL immunochip assay for TSH

The FL immunochiop assay system for TSH consists of an anti-TSH-monoclonal Ab (mAb) and chicken IgY coated strip in a disposable chip (Figure 1), a detection buffer containing FL-labeled anti-TSH-polyclonal Ab (pAb)/FL-labeled anti-chicken IgY, a calibration chip, and a laser *i*-CHROMA^{TM} scanner.

When the detection buffer is mingled with human blood sample in a tube, the FL-labeled anti-TSH-pAb (detector Ab) interacts with TSH in blood. This mixture is then loaded onto the sample well on the immunochip and migrates through the nitrocellulose membrane matrix of the test strip. As migrating, the FL-labeled anti-TSH-pAb/TSH complexes bind to the immobilized capture anti-TSH-mAb at the test line of the strip. The capture mAb recognizes a different antigenic site of TSH from detector Ab. The complexes are accumulated at the test line during incubation. Thus, the more TSH is in blood sample, the more the complexes are accumulated at the test line on the matrix of the chip. Upon inserting sample-loaded immunochip into *i*-CHROMA^{TM} scanner, the laser light illuminates the membrane window of the test immunochip thereby triggering FL from the FL-labeled anti-TSH-pAb/TSH complexes accumulated at the test line. The FL intensity is directly proportional to the concentration of TSH in sample and the amount of TSH in sample is determined from a stored, multipoint calibration curve.

Figure 1. The representative appearance of FL immunochip for TSH. Scanning window (left oval circle), sample loading well (right round circle) and insertion arrow into scanner are seen from the top panel of the immunochip. The nitrocellulose membrane strip immobilized with anti-TSH-mAb and chicken IgY is lied on the bottom panel. Sizes of strip and immunochip, and amounts of Abs immobilized were described in details at the section of strip, immunochip, and scanner in Materials and Methods.

The developed FL immunochip assay system for TSH was tested over a wide range of TSH concentrations (0-100 μIU/mL). Figure 2A was real FL images depending on various concentrations of TSH in samples. It clearly showed that there were increased FL intensities at test lines but no changes of FL intensity at control line as TSH concentration increased from 0 to 100 μIU/mL in the sample. Figure 2B was a profile of relative FL units (RFUs) at the test and control lines when the immunochips of Figure 2A were scanned in *i*-CHROMA^{TM} scanner according to a standard assay procedure. The RFUs of the test lines (left peak) increased gradually as the concentrations of TSH increased. In contrast, the RFUs at the control lines (right peak) remained constant at different concentrations of TSH.

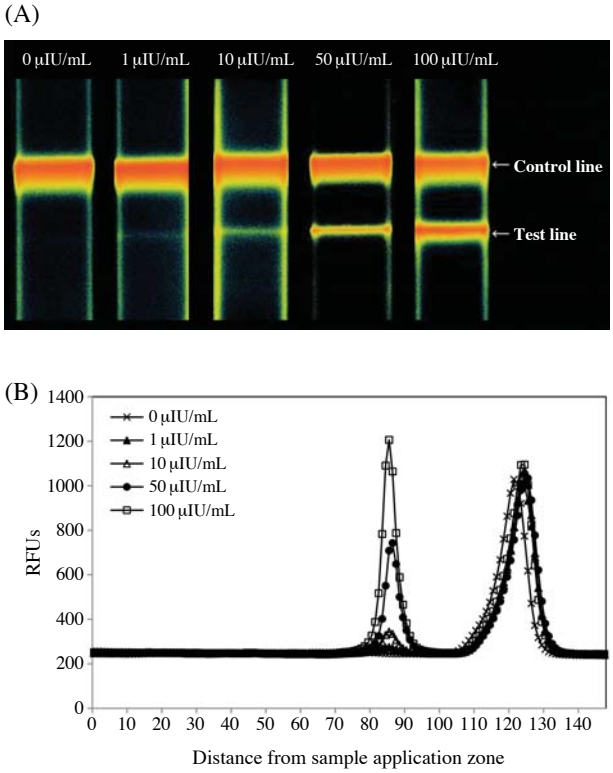

Figure 2. The real FL images at various concentrations of TSH (A) and the scanning profiles of relative FL units (RFUs) in *i*-CHROMA^{TM} TSH assay system (B). A is the real FL images of immunochip taken in 15 min after the mixture of sample and detection buffer is loaded onto the well of immunochip by GenePix 4300 scanner. In B, the real FL images in A were transformed to a profile of RFUs at the test and control lines as the immunochips were scanned in *i*-CHROMA^{TM} scanner. The RFUs were plotted on the *Y*-axis and an arbitrary distance from the sample well of immunochip was plotted on the *X*-axis.

The scanning profile of FL intensity was consistent with real FL images of Figure 2A, and also indicating that the interaction between FL-labeled anti-chicken IgY and chicken IgY was independent of TSH concentrations in samples and thus functioned as a good internal standard.

For the calibration curve, the RFUs displayed at the test and control lines were converted into the area values (test: A_T, control: A_C), and the area ratios (A_T/A_C) were plotted against the concentrations of TSH. A reliable correlation coefficient (r) was observed between the area ratio value and the TSH concentration (R=0.996), and the expected linearity was displayed throughout the entire tested TSH range, as shown in Figure 3. CVs fell between 2.8 and 9.6% for 10 independent experiments at different concentrations of TSH. The limit of detection for the assay system was

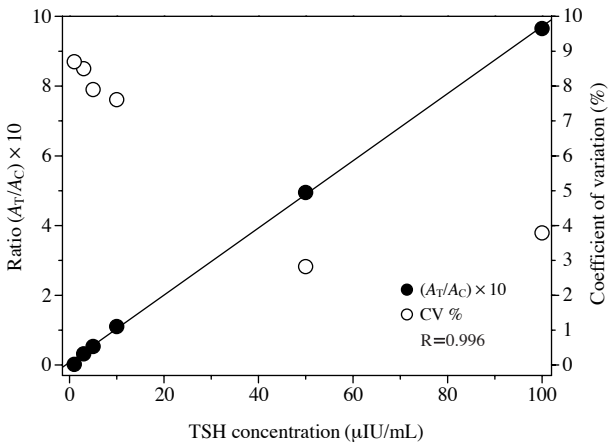

Figure 3. The calibration curve obtained from the area ratio (A_T/A_C) against the concentration of TSH. The Pearson coefficient correlation (r) between the area ratio (A_T/A_C) and TSH concentration was 0.996 ($p<0.001$) and CVs at various tested TSH concentrations fell between 2.8% and 9.5%. The spiked points for calibration curves (●) and for CVs (○) were obtained from the mean values of 10 independent experiments at each TSH concentration.

1 μIU/mL of TSH, calculated as mean value plus 3 SD of a zero calibrator. The *i*-CHROMA^{TM} TSH assay system did not show prozone or hook effect even at a TSH level of 2,500 μIU/mL. A higher level than 100 μIU/mL of serum TSH does not have an important clinical meaning and thus the working dynamic range of the FL immunochip TSH assay system was determined to be 1-100 μIU/mL.

Although the clinical cut-off value of TSH is not constant by depending on the underlying cause and the population studied, normal serum TSH concentration is between 0.4 and 4.0 μIU/mL[14] and TSH level of clinical hypothyroidism and hyperthyroidism is >10 μIU/mL[15] and <0.02 μIU/mL[16], respectively. Most current automated methods as a third-generation assay are based on nonisotopic immunometric principles and their functional sensitivity for TSH assay is <0.01 μIU/mL to detect primary hyperthyroidism[16]. Thus, the functional sensitivity of FL immunochip assay method was not comparable with those of automated reference methods. But considering dynamic working range and CV, the new FL immunochip assay system demonstrated successfully quantified serum concentrations of TSH to distinguish primary hypothyroidism and normal serum TSH.

Analytical performances of fluorescence immunochip assay for TSH

The interference, recovery, and imprecision tests described below were carried out to evaluate the techni-

Table 1. Interference tests of fluorescence immunochip assay system.

Interference substances	Interference substances conc.	Mean value* (μIU/mL)	Expected value (μIU/mL)	Recovery (%)
D-glucose	600 mM/L	2.38	2.35	101.2
		11.74	11.62	101.1
		22.51	22.22	101.3
L-Arscorbic acid	2 mM/L	2.38	2.35	101.2
		11.72	11.62	100.9
		22.32	22.22	100.4
Bilirubin (unconjugate)	4 mM/L	2.36	2.36	100.0
		12.07	11.30	106.8
		21.53	21.66	99.4
Hemoglobin (human)	20 g/L	2.37	2.35	100.8
		11.83	11.62	101.8
		22.38	22.22	100.7
Cholesterol	130 mM/L	2.45	2.44	100.5
		12.63	12.24	103.2
		22.12	22.66	97.6

*Mean value of 5 replicates

Table 2. Recovery test of fluorescence immunochip assay system.

i-CHROMA TSH Whole Blood (μIU/mL)			
	Measured concentration*	Expected concentration	Recovery (%)
100%	62.52	62	99.2
50%	31.41	31	98.7
25%	15.06	15.5	102.9
12.50%	8.14	7.75	95.2
6.25%	3.99	3.88	97.2
3.12%	2.01	1.94	96.5
1.55%	1.04	0.97	93.3
0%	< 1.0	0	–

* Mean value of 20 replicates

Table 3. Imprecision tests of fluorescence immunochip assay system.

Conc. (μIU/mL)	Intra assay			Inter assay		
	Mean value*	SD	CV (%)	Mean value*	SD	CV (%)
3.5	3.67	0.35	9.58	3.64	0.33	9.13
16	16.64	0.56	3.35	16.02	0.56	3.51
35	34.04	1.51	4.42	32.48	1.44	4.45

*Mean value of 20 replicates

cal performance of the FL immunochip assay system for TSH.

The FL immunochip assay for TSH exhibited no significant interference from common interfering substances in serum. As shown in Table 1, five interfering substances were tested at concentration beyond their physiological levels: bilirubin (4 mM/L), hemoglobin (20 g/L), glucose (600 mM/L), L-ascorbic acid (2 mM/L), and cholesterol (130 mM/L). None had a significant impact on the results of the assay when compared to those without interfering substances. The cross-reactivities were also tested with bio-molecules that have similar structures to TSHβ. A large amount of LHβ, FSHβ, and hCGβ, such as 1,000 mIU/mL, 1,000 mIU/mL, and 200,000 mIU/mL, respectively, were added to the test sample (s). However, the results of the FL immunochip assay test did not show any significant cross-reactivity with these biomolecules (data not shown).

Samples were prepared from two serum controls for a recovery test. The TSH levels of high and low control solution were 62 μIU/mL and TSH-free-solution, respectively. The high control (100%) was diluted with the low control (0%) to the following final percentages: 100%, 50%, 25%, 12.5%, 6.25%, 3.12%, 1.55% and 0%. Twenty replicates in one analytical run were tested at each TSH level. Table 2 showed the test result of the comparison between the measured and the expected values of TSH. A mean recovery of control was 97.6% with a linearity maintained throughout the measuring range (r=0.999), which was consistent with the result obtained from the calibration curve of Figure 3.

The imprecisions of the intra- and the inter-assay were evaluated to determine the accuracy of *i*-CHROMA™ TSH assay. The intra-assay was performed on 20 replicate tests for each TSH concentration of 3.5, 16, and 35 μIU/mL. The inter-assay was carried out on 10

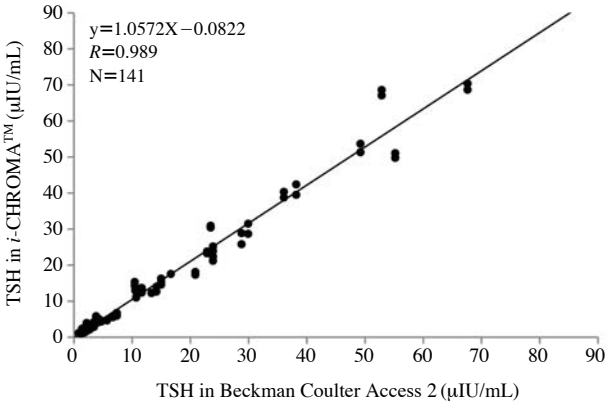

Figure 4. Comparison of the TSH levels in blood samples between Beckman Coulter Access 2 analyzer (*X*-axis) and *i*-CHROMA™ (*Y*-axis) FL scanner. 141 samples were analyzed for TSH levels side-by-side and simultaneously. 50 μL of whole blood with *i*-CHROMA™ immunochip assay and 30 μL of serum with reference assay were used for comparison of TSH concentration. The Pearson coefficient correlation (*r*) between two assay methods was 0.989 ($p < 0.001$).

sequential days, two runs per day, with 20 replicates at each concentration. The intra and inter-assay CVs in the FL immunochip assay were, respectively, 9.58% and 9.13% at 3.5 μIU/mL, 3.35% and 3.51% at 16 μIU/mL, 4.42% and 4.45% at 35 μIU/mL, as shown in Table 3. These results suggested that the developed TSH assay system was comparable to automated reference assays with CVs < 10% in the dynamic range[17].

Comparability of fluorescence immunochip assay to a reference assay

The developed FL immunochip assay system for TSH was compared with an automated reference assay analyzer being widely used: the alkaline phosphatase enzyme linked assay on Beckman Coulter Access 2. The levels of TSH of 141 blood donors were measured at the same time and analyzed in each system according to each specific instruction manual. The whole blood and the serum sample were used, respectively, in *i*-CHROMA™ immunochip assay and in Beckman Coulter Access 2 assay. The result of comparison plot between *i*-CHROMA™ TSH assay method and the reference assay method was shown in Figure 4, and there was a highly significant correlation between two assay methods with $r=0.989$ ($p < 0.001$). Therefore, this test result demonstrated that the FL immunochip TSH assay provided a confident performance that paralleled other current system in use.

As shown in reference assay system, many commercial automated analyzers employing immunological methods have limitations on sample application or quantitative single sample analysis[18]. Many samples should be collected to carry out one test and the test result would not be come out even in 30 min after the assay starts. The rapid test kit can use for a single sample analysis but can be applicable only for qualification[19]. The FL immunochip TSH assay has some advantages over automated reference assays or the rapid test kit in that it has a POCT platform by using whole blood and can measure TSH concentration with fast turnaround time of 15 min by applying a single sample.

In conclusion, the *i*-CHROMA™ TSH assay method exhibited good analytical performances in terms of linearity, recovery, interference, imprecision, and the test result of TSH concentration was comparable to commercially available automated third-generation immunometric reference analyzer. The developed FL immumochip assay is the only method that can quantify TSH concentration in whole blood. It meets the criteria of POCT, including affordable cost, a disposable device, and requiring minimum maintenance to perform test.

Materials and Methods

Materials

Anti-TSH-pAb and anti-TSH-mAb were purchased from Biocheck, Inc. (Foster city, CA, USA) and anti-chicken IgY Ab was come from Jackson Immuno-Reaserch Laboratories, Inc. (West Grove, PA, USA). Human TSH was from NIBSC (Hertfordshire, UK). Sephadex G25 and activated Alexa Fluor 647 were obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA) and Molecular Probes (Eugene, OR, USA), respectively. Nitrocellulose membrane was purchased from Millipore (Watertown, MA, USA), sample pad and the absorption pads were obtained from Schleicher and Schuell (Keene, NH, USA).

Labeling of antibodies with fluorescent dye

For conjugation of the anti-TSH-pAb with a FL-dye, 10 μL of a 1 mol/L sodium bicarbonate buffer (pH 8.3) was mixed with 100 μL of anti-TSH-pAb (1 mg/mL) in phosphate-buffered saline (1 × PBS) and followed by the addition of 1 μL of activated Alexa Fluor 647 (10 mg/mL) to the mixture. After overnight incubation at 4°C, the mixture was applied onto a Sephadex G25 column to remove the free dye, and FL-labeled pAb conjugates were collected as elutes after centrifugation of the column at 2,500 rpm (Model 5810R, Eppendorf) for 2 min. The anti-chicken IgY was similarly conjugated with same FL chromogen and purifi-

ed with same processes as noted above. The FL-labeled anti-TSH-pAb and the FL-labeled anti-chicken IgY were mixed together with the assay buffer to form the detection buffer and were kept at 4°C until use.

Strip, immunochip, and scanner

The FL-ICA test strip was fabricated in-house to fit into a disposable chip and a laser FL scanner. The sample pad and the absorption pad were cut to a size of 4 × 20 mm and assembled with anti-TSH-mAb and chicken IgY coated NC onto a polystyrene backing card. The capture mAb and the control IgY were dispensed as 1-mm-wide lines at the test line and the control line, respectively, using a BioJet dispenser (BioDot, Irvine, CA, USA). The assembled strip was kept in a dry vacuum chamber overnight before being placed into a chip (15 × 90 mm) (Figure 1). The chip was then sealed in a foil pouch containing a desiccant and stored at room temperature. Because the appearance of the test chip was unique, a laser FL scanner called *i*-CHROMA™ (Boditech Med, South Korea) was used to measure the distribution of FL intensity along the strip of chip. The principle of *i*-CHROMA™ FL scanner was previously described in detail[20].

Assay procedure

The detection buffer was a mixture of FL-labeled pAb and FL-labeled anti-chicken IgY (internal control Ab) in 1 × PBS. A quantity of 50 μL from whole blood was added to 100 μL of detection buffer, and 100 μL of the mixture was then loaded onto the sample well of chip. After 15 min of incubation for immune reactions, the chip was inserted into the laser FL scanner for detection of FL intensity. The scanner converts FL intensity to numeric data, calculates the relative amount, and displays the level of TSH in the sample as μIU/mL on the screen.

Blood sample

Blood samples were obtained from individuals who visited the Kangwon National University Medical Center in Chuncheon, South Korea. Informed consent was obtained from volunteers before their participation in the study. Venous blood was collected in 5 mL vacuum tubes (Becton-Dickinson, Franklin Lakes, USA) containing heparin. The blood samples were generally tested within 30 min of collection. For serum collection, the blood was collected in tubes not containing anticoagulants, leaving to settle for 30 min for blood coagulation, centrifuged at 3600 × g for 10 min at 4°C, and then aliquoted in small volumes and frozen at −70°C before analysis.

Method of comparison and statistics

The concentrations of TSH in blood samples were measured by *i*-CHROMA™ TSH assay system and were compared with those obtained by Beckman Coulter Access 2 analyzer. MedCalc version 7.6 software (Mariaekerke, Belgium) and Microsoft Excel 2010 (Redmond, WA) were used for analysis and comparison of test results. Pearson correlation coefficients (r) and linear regression with the least-squares method were used to evaluate correlations between methods. P values < 0.05 were considered significant.

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