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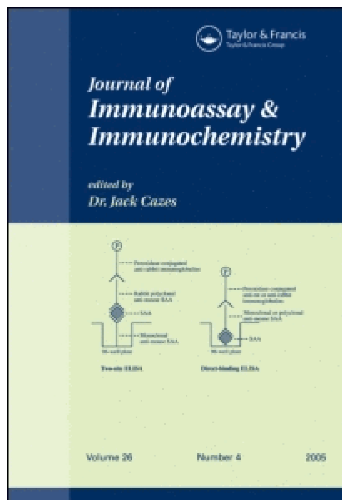

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DEVELOPMENT OF AN IMMUNOENZYMOMETRIC ASSAY (IEMA) FOR THE ESTIMATION OF HUMAN THYROID STIMULATING HORMONE (hTSH) IN SERUM

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DEVELOPMENT OF AN IMMUNOENZYMOMETRIC ASSAY (IEMA) FOR THE ESTIMATION OF HUMAN THYROID STIMULATING HORMONE (hTSH) IN SERUM

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□ *A sensitive immunoenzymometric assay (IEMA) of serum thyrotropin (hTSH) was developed using anti-hTSH rabbit polyclonal antibody and anti-hTSH in-house monoclonal antibody with a sensitivity of 0.12 mIU/L. Serum samples were incubated in ELISA wells precoated with polyclonal antibody. The hTSH bound to the wells was incubated with monoclonal antibody (detector antibody) and further with goat anti-mouse antibody–horse radish peroxidase (GAM-HRP), which obviates the need to label the detector antibody. The assay was validated by recovery, linearity, and cross-reactivity experiments with a working assay range of 0.15 to 100 mIU/L and $<10\%$ coefficient of variation (CV) for both intra- and interassay. Good correlations were obtained when compared with Immunotech hTSH IRMA ($r = 0.971$, $n = 35$). This in-house ELISA can be used as an initial screening test for thyroid dysfunction.*

Keywords hTSH, hybridoma technique, IEMA, monoclonal antibody, polyclonal antibody, serum

INTRODUCTION

Thyroid stimulating hormone (hTSH) is a glycoprotein (28KDa) secreted by thyrotrope cells of the anterior pituitary gland. hTSH is composed of two non-covalently bound, distinct subunits designated as α and β.[1–3] The α subunit is common to the other glycoprotein hormones, such as follicle stimulating hormone (FSH), leutinizing hormone (LH), and chorionic gonadotropin (CG). The β subunit determines both the biological and immunological specificity.[4] The selection of an appropriate pair of antibodies against hTSH to have maximum specificity is a crucial task.

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The main function of hTSH is the regulation of synthesis and release of the thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) by a negative feedback effect on the target organ, the thyroid.[5] Measurement of serum thyrotrophic (TSH) is being advocated for first-line assessment of thyroid function, both in screening for suspected new thyroid disease and the monitoring of thyroxine therapy in patients with existing thyroid disease.

Two-site immunoenzymometric assay (IEMA)[7] for hTSH makes use of two antibodies directed against two different epitopes. One of the antibodies is immobilized to the solid phase, and the other is labeled directly or indirectly (e.g., monoclonal antibody–rabbit anti-mouse–HRPO) with an enzyme. Both the antibodies bind simultaneously to hTSH present in the calibrator, patient sample, or controls. The sandwich formed remains bound to the solid phase antibody, and the activity of the enzyme present on the solid phase is well quantified by reaction with a suitable substrate to produce color. The IEMA standardized is a second-generation assay, which provides a means for discrimination between hyperthyroid–euthyroid range. It has a functional sensitivity ($<10\%$ between assay CV) of 0.12 mIU/L.

In this article, the generation of a monoclonal antibody against hTSH using hybridoma technology[8,9] along with the standardization of two-site IEMA[10] is described. In this assay, the hTSH polyclonal antibody is used as a solid-phase antibody and in-house monoclonal antibody as the detector antibody, such that the detector antibody is indirectly labeled with an enzyme by incubating the system with goat anti-mouse–HRP conjugate. Generation of monoclonal antibodies in our laboratory included immunization, fusion, screening, and purification.

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EXPERIMENTAL

Materials

BALB/c mice were obtained from BARC (Bhabha Atomic Research Centre, Mumbai, India) animal facility. The SP2/0 Ag-14 myeloma cell line was obtained from Radiation Medicine Centre, Mumbai, India. Polyethylene glycol (PEG) - 4000, HAT (hypoxanthine, aminopterine, thymidine) medium, Complete Freunds Adjuvant (CFA), Incomplete Freunds Adjuvant (IFA), Pristane (2,6,10,14-tetramethyl pentadecane) was obtained from Sigma (St. Louis, Missouri, USA). Protein-A sepharose was obtained from Pharmacia (Uppasala, Sweden). hTSH (standard grade) was obtained from ICN biomedicals (Cleveland, Ohio, USA). hTSH rabbit polyclonal antibody was procured from M.P. Biomedicals (Solon, Ohio, USA). ELISA microtiter plates were obtained from Nunc (Rosklide, Denmark). GAM-HRP (Goat anti mouse-HRP) and TMB/H_2O_2 (Tetramethylbenzidine/hydrogen peroxide substrate was procured from Bangalore

Genei (Bangalore, India). Milk protein was procured from Nestle (Mumbai, India). Tween-20 was procured from Merck (Mumbai, India). The 96 well ELISA auto reader was obtained from Tecan Spectra (Tecan Austria, Groedig, Austria). Comparative validation studies were carried out using hTSH IRMA kit from Immunotech (Praha, Czech Republic).

The following buffers were used:

- Coating buffer: 0.1 M $NaHCO_3$ buffer pH 8.6
- Blocking buffer: 0.1% milk protein in 0.05 M phosphate buffer pH 7.4
- PBS: phosphate buffered saline
- Wash buffer: PBS containing 0.2% BSA and 0.1% tween 20
- Reaction terminating solution: 2M H_2SO_4

Generation of hTSH Monoclonal Antibodies

Immunization

BALB/c mice were immunized intraperitoneally (ip) with 10 μg of hTSH in 200 μL of phosphate buffered saline (PBS) emulsified in 200 μL of complete Freund's adjuvant. Mice were boosted ip at three-week intervals with 10 μg of hTSH in 200 μL of PBS emulsified at 1:1 with incomplete Freund's adjuvant. Mice showing high serum titers of hTSH antibody in the test bleed were given final intravenous boosters with 20 μg of hTSH in saline. On the third day, these mice were sacrificed to obtain spleen cells for fusion with myeloma cells.

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Monoclonal Antibodies: Generation and Purification

The fusion of the spleen cells with the myeloma cells SP2/0-Ag-14 was carried out using PEG-4000. These fused cells were grown in HAT medium for the selection of hybrid clones. The clones were screened for antibodies against hTSH. The supernatant from the wells containing the growing clones was added to the hTSH coated microwells and incubated. After washing the microwells, GAM-HRP conjugate was added and incubated. After washing, TMB/H_2O_2 substrate was added. The presence of antibody was indicated by the appearance of color as measured by OD (Optical Density) at 450 nm. The selected hybridoma clones were subcloned twice by limiting dilution over a BALB/c peritoneal macrophage feeder layer. The monoclonal antibodies were produced in large amounts by injecting hybridoma cells ip into BALB/c mice primed with pristane. These antibodies were purified from the ascitic fluid by Protein A chromatography. Three monoclonal antibodies (M_1, M_2, and M_3) were evaluated for use in the hTSH IEMA.

Preparation of Working Standards

hTSH of known potency obtained from commercial source was used for the preparation of standards. The standard hormone from the stock was dissolved in the buffer according to the manufacturer's recommendation to prepare 2.5 IU/mg. The stock standards were calibrated against the WHO Reference Standard (Second IRP 80/558) before preparation of working standards. Horse serum was used as a diluent after testing it for absence of TSH. Standard concentrations ranging from 0.15–100 mIU/L were made in horse serum using the above calibrated standards, aliquoted, and stored frozen at −20°C.

Optimization of Assay

Identification of Matched Pair of Antibodies

A 200-μL solution containing 1 μg each of the three hTSH monoclonal antibodies (M_1, M_2, and M_3) and 200 μL of 1:1000 dilution of hTSH polyclonal antibody (P) in coating buffer was added to four sets of microwells in duplicate and incubated overnight at 4°C. After washing with wash buffer, the microwells were blocked with 1% milk protein and washed again. 200 μl of hTSH (100 mIU/L) in PBS was added to the washed microwells and incubated at 37°C for 2 h. After washing, 2.5 μg/200 μL of the three monoclonal antibodies (M_1, M_2, and M_3) were added as detector antibodies to each set and the wells, which were further incubated for 2 h at 37°C. To the washed microwells, 200 μL of GAM-HRP was added and incubated at 37°C for 2 h. TMB/H_2O_2 substrate (200 μL) was added to each well after washing, and the color was developed by incubating for 10 min in the dark. The reaction was terminated by the addition of the terminating solution, and the resulting OD was read at 450 nm in the ELISA reader. To determine the nonspecific binding, the reagents were incubated in antibody uncoated wells.

Optimization of Assay Using the Identified Pair

Using the identified matched pair of antibodies, various reaction parameters such as concentration of reagents, reaction kinetics, sequence of addition of the reagents, time, and temperature were evaluated to arrive at a suitable assay system.

Briefly, the assay protocol formulated after optimization of reaction parameters consists of adding the reagents in the following sequence. The standard/sample (200 μL) was added to the solid phase antibody (polyclonal antibody P)–coated microwells and incubated at 37°C for 2 h. After

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washing, 200 μL containing 1 μg of detector antibody (monoclonal antibody M_2) was added and incubated at 37°C for 2 h. To the washed microwells, 200 μL of GAM-HRP (1:500) was added and incubated at 37°C for 2 h. The microwells were washed, and 200 μL of TMB:H_2O_2 (1:20) was added and incubated for 10 min in the dark. The reaction was terminated by adding 50 μL of the terminating solution, and the OD was read at 450 nm.

In order to reduce the number of assay steps, microwells were precoated with the identified capture antibody (1:1000). The stability of these precoated microwells was studied over a period of time at 4°C. The developed assay was subjected to quality control tests for precision, sensitivity, specificity, reproducibility, recovery, and parallelism.

RESULTS

After the fusion experiments, 384 growing hybrid clones were observed. Upon screening for hTSH-positive clones in ELISA, it was observed that among these, a large number of clones showed hTSH positivity.

hTSH-positive antibodies were screened for cross-reactivity against hCG (2000 mIU/mL), hLH (300 mIU/mL), and hFSH (1500 mIU/mL) in ELISA. Ten hTSH-positive clones showing <0.2 OD for the cross-reactants were pooled for carrying out the subcloning by limiting dilution. Nine hTSH-positive clones were obtained after limiting dilution, out of which, the monoclonal antibodies secreted by 3 clones (M_1, M_2, and M_3) did not show significant cross reactivity with hCG (2000 mIU/mL), hLH (300 mIU/mL), and hFSH (1500 mIU/mL). Therefore these three monoclonal antibodies along with hTSH polyclonal antibody (P) as pairs were tested for cross-matching for simultaneous binding to hTSH for use in two-site hTSH IEMA.

In the identification of the matched pair of antibodies, as shown in Table 1, the highest OD of 2.4 was obtained with the pair having polyclonal antibody (P) as the solid phase antibody and M_2 as the detector antibody.

TABLE 1 Identification of Matched Pair of Detector and Capture Antibody

	Mean OD at 100 mIU/L of hTSH Standard (n = 5)				
Detector Antibody	SM_1	SM_2	SM_3	SP	Uncoated Wells
M_1^*	0.61 ± 0.01	0.65 ± 0.02	0.62 ± 0.01	0.80 ± 0.03	0.05 ± 0.01
M_2^*	0.57 ± 0.01	0.62 ± 0.01	0.55 ± 0.01	**2.40** ± 0.1	0.05 ± 0.01
M_3^*	0.70 ± 0.03	0.56 ± 0.02	0.61 ± 0.01	0.63 ± 0.01	0.04 ± 0.01

M^* – Detector monoclonal antibody; SM_1, SM_2, SM_3 – Solid phase monoclonal antibodies; SP – Solid phase polyclonal antibody.

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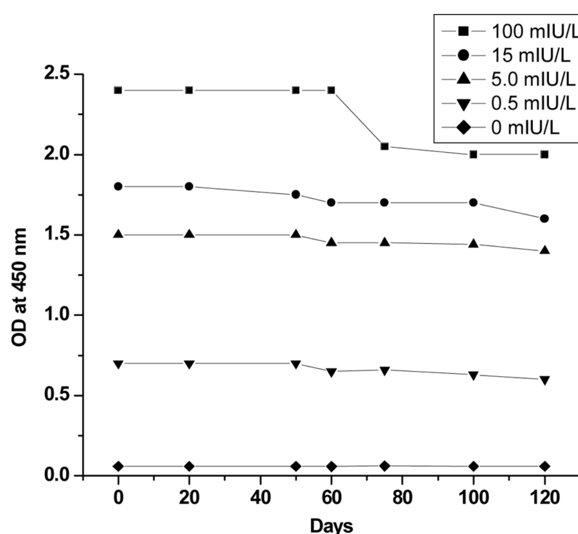

FIGURE 1 Stability studies of the capture antibody coated microwells.

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The nonspecific binding was very low (OD 0.05), which shows that the assay reagents are not nonspecifically bound to the microwells. This pair was used for the optimization of hTSH IEMA.

The precoated microwells were found to be stable for a period of up to six months, after which there was decrease in the binding with hTSH as reflected in the decrease in OD values, as shown in Figure 1.

The assay procedure was optimized for the estimation of hTSH, and the optimized parameters such as concentration of reagents, reaction kinetics, incubation conditions, and sample volume are given in Table 2. The flow chart of the assay is given in Figure 2. The optimized assay gave a low binding at hTSH standard concentration of 0 mIU/L (OD 0.05), a maximum binding at highest standard concentration (100 mIU/L) of OD 2.4, and the highest sensitivity index (difference between 0 and next standard).

The average of ODs obtained in 20 different assays over a period of 32 months is given in Table 3 and Figure 3. For calculating the sensitivity of the assay, the dose–response curve was constructed on linear graph paper

TABLE 2 Optimized Assay Parameters

Factor/Parameter	Optimized Parameter
Titer of capture antibody (P)	1:1000
Standard/sample volume	200 μL
Concentration of detector antibody (M_2)	2.5 μg/mL
Titer of GAM-HRP conjugate	1:1600
Titer of substrate (TMB/H_2O_2)	1:20

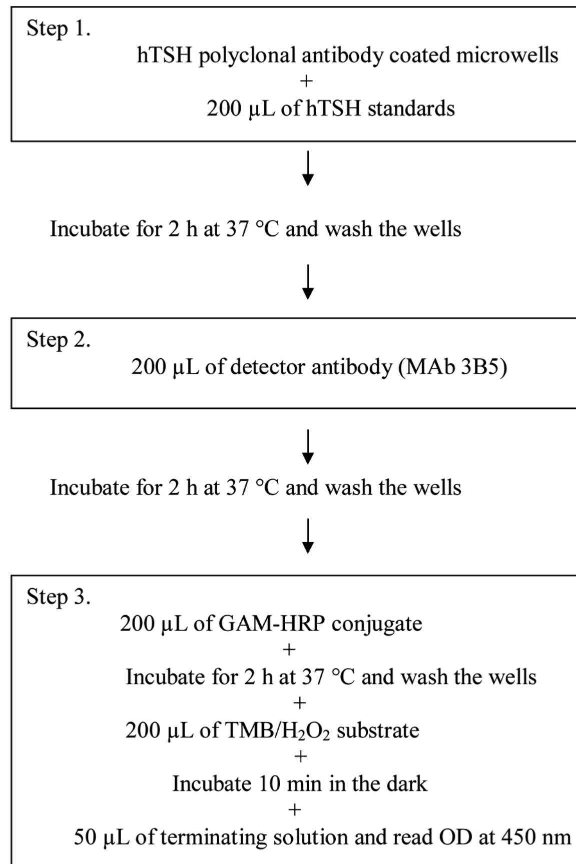

FIGURE 2 Optimized protocol for hTSH IEMA.

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with mean response values. The standard deviation involved in the measurement of zero response was calculated from a set of 10 values of response at zero antigen concentration. The dose corresponding to a

TABLE 3 Standard Curve Data for 20 Assays*

hTSH (mIU/L)	OD at 450 nm ($x \pm 2$ S.D.)
0	0.05 ± 0.02
0.15	0.4 ± 0.15
0.5	0.7 ± 0.25
1.5	1.0 ± 0.4
5.0	1.4 ± 0.3
15	1.7 ± 0.3
50	2.0 ± 0.2
100	2.4 ± 0.2

*For 20 assays, using different batches of reagents over a period of 32 months.

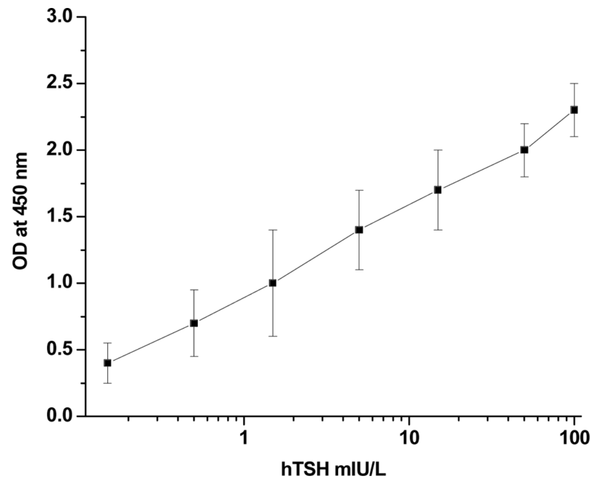

FIGURE 3 Optimized hTSH IEMA standard curve.

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response two standard deviations away from the mean zero dose response (to the upper side) was the minimum amount, which gave a response statistically different from zero dose response, at a 95% confidence level. This value was taken as the sensitivity of the assay system.

The sensitivity of the assay, estimated as the minimum dose that can be measured with an error less than 10%, was observed to be 0.12 mIU/L. The intra-assay precision was determined by replicate analysis of two control serum samples in a single assay, and interassay precision was estimated by

TABLE 4 Characteristics of IEMA for hTSH

Parameter	hTSH IEMA
NSB	0.06
Sensitivity	0.12 mIU/L
Assay range	0.12–100 mIU/L
Recovery	93–109%
Dilution test	90–101%
Intra-assay variation	Control A range (3.04–4.76 mIU/L)
	Mean $x = 4.0$ mIU/L, % CV = 4.7
	Control B range (12–18 mIU/L)
	Mean $x = 15$ mIU/L, % CV = 3.0
Interassay variation	Control A range (3.04–4.76 mIU/L)
	Mean $x = 5.0$ mIU/L, % CV = 10
	Control B range (12–18 mIU/L)
	Mean $x = 14.8$ mIU/L, % CV = 10
Hook effect	No effect up to 250 mIU/L
Cross-reactivity	hLH (300 mIU/mL) $< 0.01\%$
	hCG (2000 mIU/mL) $< 0.01\%$
	hFSH (1500 mIU/mL) $< 0.002\%$

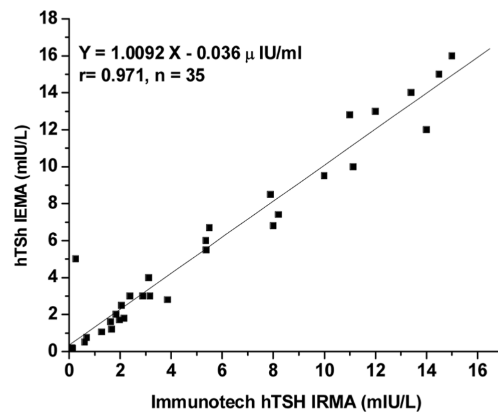

FIGURE 4 Correlation between the developed hTSH IEMA and commercial hTSH IRMA.

duplicate measurement of the same control serum samples in 20 different runs. Dilution linearity of the assay was evaluated by assaying samples serially diluted with hTSH-free serum. A good agreement between the measured and expected values was observed. The analytical recovery of the known concentrations of hTSH added to the aliquots of 10 serum samples lies within acceptable limits. All the validation results are tabulated in Table 4.

Comparison of levels of hTSH in different serum samples estimated by the present method and by using commercially available IRMA coated tubes (Immunotech), exhibited good agreement. Regression analysis showed good correlation ($r = 0.971$, $n = 35$) with commercially available

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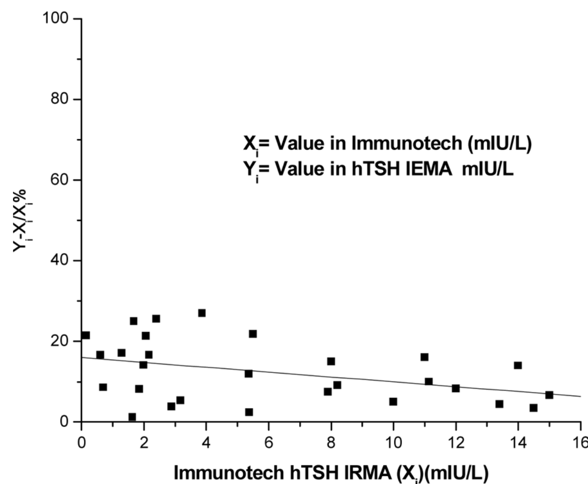

FIGURE 5 Percent difference between the two assays plotted against the comparative method (IRMA assay from Immunotech).

kits ($y = 1.0092$, $x = 0.036$ mIU/L) for hTSH as shown in Figure 4. Figure 5 shows the percent difference between the two assays should be shown where $\%(y_i - x_i)/x_i$ plotted against the comparative method (IRMA assay from Immunotech).

DISCUSSION

Considering the number of positive clones obtained, the hTSH molecule can be considered to be adequately immunogenic in mice. As the percentage of non-cross–reactant clones is less, it can be concluded that the alpha subunit of the molecule plays a significant role in the immunogenic potency of hTSH. The immunodominant[11] region of hTSH is clustered at the interface of the α and β subunits. Most monoclonal antibodies are generated against the epitopes of this region. This makes it difficult to get the right combination of two monoclonal antibodies against epitopes, which are spaced far apart for the development of sandwich assays such as immunoenzymometric assays. Also, during the labeling process, if the paratopes of the selected monoclonal antibodies get labeled, then these monoclonal antibodies will not be able to bind to their respective epitopes. In order to avoid this, we have not labeled the detector monoclonal antibody; rather the GAM-HRP detects this monoclonal antibody in this assay. Therefore in this indirect ELISA, the immunoreactivity between the detector monoclonal antibody and the hTSH is not disturbed.

The hTSH estimation is a very sensitive indicator for the diagnosis of hypothyroidism and hyperthyroidism, and the diagnostic thyroid profile is not complete without the hTSH estimation; hence the development of a specific and sensitive hTSH IEMA is essential. The third-generation serum assays are more sensitive than the second-generation assays. However the reagents are quite expensive, so the use of expensive third-generation assays instead of a more economical second-generation assay[6,12] is not justified. In addition, second-generation assays do provide the required assay sensitivity for clinical purposes.

Due to the structural homology of the different glycoprotein hormones with hTSH, and also the need to obtain a matched pair of antibodies, which can bind simultaneously to the analyte without causing steric hindrance to each other, the selection of the best-matched pair of antibodies is a challenging step for the development of a specific and sensitive assay. Although the antibodies used in this study were against different epitopes on hTSH, it is essential to identify the appropriate combination of the best-matched pair with respect to the performance characteristics of the immunoassay.

The performance of matched pairs in terms of binding and sensitivity index were quite different between the different combinations of antibody

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pairs. This may be due to the fact that the binding sites of these antibodies may overlap or lie very closely adjacent, resulting in steric hindrance. Hence, selection of compatible sandwich partners, careful optimization of reagent preparatory methods, reaction conditions, and concentrations are necessary in the development of a user-friendly and robust IEMA. In the present study, a simple and sensitive immunoenzymometric assay has been developed based on hTSH monoclonal antibody generated in-house and a commercial polyclonal capture antibody pair for the estimation of hTSH in human serum.

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