Rapid One-Step Modified Technique in the Evaluation of Thyroglobulin by the Immunoradiometric Assay (IRMA) for Monitoring Differentiated Thyroid Carcinoma Patients

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ABSTRACT

Objective: The aim of this study was to develop a modified one-step technique for measuring serum thyroglobulin (Tg), which is currently used as a tumor marker for differentiated thyroid carcinoma (DTC) by using the immunoradiometric assay (IRMA) of the THYROGLOBULINE IRMA kit as compared with the standard method.

Methods: The surplus serum specimens from 111 DTC patients who had been treated and followed up at the Division of Nuclear Medicine, Department of Radiology and 105 healthy donors at the Department of Transfusion Medicine, Faculty of Medicine, Siriraj Hospital were included. The technique for serum Tg measurement was optimized from the two-step IRMA standard kits to the one-step modified method.

Results: The one-step modified technique results in a decrease of turnaround time (TAT) from two days of both one-step and two-step gold standard kits to only two hours. The optimal volume of ^{125}I-TgAb was found to be the same that of the kit's technique. The optimal time for incubation was decreased from overnight at 17-18^{o}C in the standard method to 90 minutes in a shaking water bath at 37^{o}C. The analytical and functional sensitivities of the modified technique were 0.28 and 1.0 ng/ml, respectively. The coefficients of variation (%CV) of both intra-assay and inter-assay precision were 0.87-4.80% and 2.87-9.75%, respectively. The accuracies of the recovery test and dilution test were 92.74-101.82% and 101.27-109.16%, respectively. No cross-reaction presented between the anti-thyroglobulin antibodies and thyroid analogue compounds. The assays working range of Tg concentration under 10% CV of precision profile was 1.59-500 ng/ml. The correlation coefficient (r) between the results of the modified technique and the two-step standard method were excellent: r = 0.99; y = 1.349x-2.421; P < 0.001; n = 111. The reference range of Tg concentration among the euthyroid healthy subjects was 0.37-20.32 ng/ml (5^{th} -95^{th} percentile).

Conclusion: The modified technique is simple, rapid, sensitive and reliable for monitoring the serum Tg level in DTC patients not only under stimulation but also during suppression therapy.

Keywords: Thyroglobulin, immunoradiometric assay, thyroid cancer

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Thyroglobulin (Tg) has been used as a tumor marker for differentiated thyroid carcinoma patients for monitoring the disease outcome after the treatment and detection of tumor recurrence. A high level of serum Tg is an indication of active residual thyroid cancer or metastatic disease. The highly sensitive method of serum Tg measurement facilitates early detection of tumor recurrence or metastasis and leads to an appropriate treatment in those patients. The original competitive radioimmunoassay (RIA) and non-competitive immunoradiometric assay (IRMA) have been used to measure serum Tg in the clinical laboratory.[1] Several investigators have developed IRMA for serum Tg measurement based on the use of monoclonal antibodies.[2-6]

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The present study aims to modify and assess this shorter turnaround time technique for serum thyroglobulin measurement in routine clinical laboratories.

MATERIALS AND METHODS

The study was conducted at the Thyroid Clinic, Division of Nuclear Medicine, Department of Radiology, Faculty of Medicine Siriraj Hospital, Bangkok, Thailand. The study protocol was approved by the Ethics Committee of the Faculty of Medicine Siriraj Hospital, Mahidol University (SI 175/2006).

Gold standard THYROGLOBULIN IRMA and TGAB ONE STEP RIA (CIS bio international, GIF-SUR-YVETTE DEDEX, France) were used to modify the effective new assay technique for serum Tg measurement. The ready for use Tg IRMA reagents were a mixture of four monoclonal anti-thyroglobulin antibodies in excess coated at the bottom of the tube, a fifth monoclonal anti-thyroglobulin - ^{125}I (^{125}I - TgAb) with a specific activity of 12 Ci/g (25 ml/vial), phosphate buffer (PB) solution pH 7.4 (35 ml/vial) and human thyroglobulin standards in PB pH 7.4 calibrated against the CRM 457 international reference (1 ml of 7 vials). The other two reagents of washing solution (25 ml/vial) and control human sera (1 ml/vial) had to be prepared before use.[6]

The two days turnaround time (TAT) of both the one-step and two-step standard method was performed according to the manufacture's instructions. Excess ^{125}I - TgAb solution was used as a tracer binding to different epitopes from those recognized by the antibodies bound to the TgAb coated tube. These antibodies were directed against the epitopic zones not recognized by the majority of anti-thyroglobulin autoantibodies present in numerous thyroid diseases. After incubating and eliminating the unbound fraction, immobilized Tg was detected by an automatic gamma counter, Wallac 1470, (Perkin Elmer, Turku, Finland).[2,6]

The in-house quality control Tg sera (mean ± 2SD, n = 20) was prepared by surplus pooled serum of low Tg (4.29-5.21 ng/ml), medium Tg (13.10-15.56 ng/ml) and high Tg (281.15-305.61 ng/ml). All pooled sera were aliquoted and stored at -20°C. The reference range of serum Tg in 105 healthy euthyroid surplus sera with TgAb-negative were provided by the Department of Transfusion Medicine, Faculty of Medicine Siriraj Hospital. Both negative (n = 60) and positive (n = 51) anti-thyroglobulin antibodies surplus sera in patients with DTC who had undergone thyroidectomy were collected at the Thyroid Clinic, Siriraj Hospital.

The modified one-step IRMA technique was optimized as follows: (1) The effect of optimal incubation time at 37°C between 3-6 hours and various volumes of ^{125}I-TgAb on the percentage of maximum and zero binding was performed by adding 100, 200, 300 and 400 ul of ^{125}I-TgAb into the ready for use monoclonal anti-thyroglobulin antibodies coated tube containing 100 ul of 500 and 0 ng/ml standard serum Tg in duplicate, respectively. (2) The effect of shorter incubation time at 37°C for 90, 120, 150, 180, and 210 minutes on 200 ul of ^{125}I-TgAb was performed by adding ^{125}I-TgAb into the monoclonal antibodies coated tube containing standard serum Tg of 0, 0.2, 1.5, 5, 15, 50, 200, 500 ng/ml and the commercial internal quality control (IQC) Tg sera of C1 (low Tg) and C2 (high Tg) calibrated against the CMR457 international reference in duplicate, respectively. (3) The effect of incubation time at 37°C for 90, 120, 150, 180, 210 minutes at various Tg sera standards of 0, 0.2, 1.5, 5, 15, 50, 200, 500 ng/ml and the commercial IQC on 400 ul of ^{125}I-TgAb was assayed.

The assay performance of the new technique was assessed as follows: (a) Cross-reactivity test (specificity test) was performed by adding the optimal volume of ^{125}I-TgAb into the TgAb coated tube in duplicate containing various concentrations of diiodothyrosine (DIT), mono-iodothyrosine (MIT), triiodothyronine (T3), thyroxin (T4) and Tg, respectively.[7] (b) Both intra-assay and inter-assay precision (%CV) was evaluated by assaying the CRM457 control Tg sera of C1, C2 and in-house Tg sera of low, medium and high concentrations. A single assay of 20 tubes of each control sample and 20 assays on different days of both in-house and commercial control sera were performed with the serum Tg standard curves in duplicate. (c) The percentage of recovery was determined by adding a pooled serum Tg specimen of 12.12 ng/ml into 7 different Tg standard concentrations of 0.2, 1.5, 5, 15, 50, 200 and 500 ng/ml. Dilution of the 2 levels of serum Tg specimen of 25.65 and 64.48 ng/ml with the CMR457 zero serum Tg standard to the ratio of 1:2, 1:4 and 1:8 was also performed. (d) The minimal detection limit or analytical sensitivity of serum Tg measurement was evaluated by within-run assaying of 40 zero standard tubes, 7 various concentrations of serum Tg standard tubes and IQC in duplicate. The sensitivity of serum Tg value corresponding to the mean count of zero point plus 2SD was estimated from the calibration curve. (e) The functional sensitivity was determined by the inter-assay precision profile at 20% CV of the lowest standard serum Tg concentration of 20 different assays including the two reagent batches.[8] (f) The new technique (y-axis) was compared to the commercially available Tg test kit (x-axis) in 111 DTC patients' sera who had been treated and followed up at the Thyroid Clinic. (g) Statistical analysis was performed by using SPSS version 10.0. Significant consideration of P value was less than 0.05. The results were presented as mean, standard deviation and %CV.

RESULTS

The percentage bounded of both 500 and 0 ng/ml serum Tg standards in duplicate antibodies coated tubes containing 200 µl ^{125}I-TgAb incubated at 37°C for 3 hours were 39.53 and 0.07%, respectively (Fig 1). Under the incubation period of 90 minutes, C1 was 19.83 ± 0.87 ng/ml and C2 was 175.18 ± 3.33 ng/ml which were higher and lower than those in the one-step standard method (17.62 ± 0.60 and 196.10 ± 6.30 ng/ml), respectively (P <0.001). After increasing the volume of ^{125}I-TgAb to 400 µl, there was no significant difference in the serum Tg levels of C1 and C2 between the standard method and the modified technique (Table 1).

The serum Tg levels of C1 and C2 measured by the modified technique using reagent batch 1 with two times bound washing showed no significant difference to those in the standard method. The comparison of C1, C2, in-house low and medium sera Tg between the two techniques was also found to not have any significant difference when using reagent batch 2 with optimal

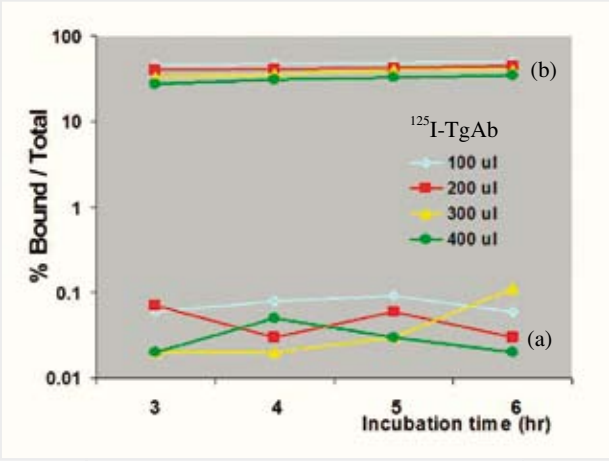

Fig 1. Percent bound of non-specific binding of standards Tg serum of 0 ng/ml (a) and maximum specific binding of 500 ng/ml (b) with various volumes of ^{125}I-TgAb in TgAb coated tubes and incubated in checking water bath at different incubation times of 3, 4, 5 and 6 hours.

three times bound washing. However, the contrary result of in-house high serum Tg was obtained (P <0.001) (Table 2). It is unnecessary to wash the bound form with the buffer four times. The optimal radioactive bound counting time of 3 minutes showed no significant difference of C1 and C2 sera Tg to those in the standard method (Table 3).

High specificity of the commercial TgAb coated tube was found. There was no cross-reactivity of the anti-thyroglobulin antibodies coated tubes to T3, T4, MIT and DIT, respectively. The analytical and functional sensitivities were 0.28 and < 1.0 ng/ml. The percentage of recovery and dilution tests were 92.74-101.82 and 101.27-102.12. Coefficient of variation (%CV) of both within-run and between-run were 0.87-4.8 and 2.87-9.75 respectively. The serum Tg reference in 105 euthyroid healthy subjects with negative TgAb was 0.32-20.39 ng/ml (5th - 95th percentile).

Good correlation exists between serum Tg levels in 111 DTC patients determined by the modified and standard method of both one-step and two-step. The linear regressions were: (a1) y = 1.272x - 7.356, (a2) y = 1.349x - 2.421, (b1) y = 1.014x + 1.124, (b2) y = 1.296x + 0.126, (c1) y = 1.696x - 12.564 and (c2) y = 1.381x - 3.114, respectively (P <0.001) (Fig 2). Serum Tg comparison between the one-step and two-step of the gold standard method also showed good correlation in the three groups of DTC patients as shown in Fig 3. Linear regression lines of groups a, b and c were y = 0.927x - 2.656, y = 0.744x + 3.734 and y = 1.226x - 6.749, respectively. The accuracies of recovery and dilution tests were 92.74 - 101.82% and 101.27 - 109.16%, respectively. The acceptable serum Tg measuring range for clinical interest of 1.59 - 500 ng/ml was obtained by using 10%CV of the precision profile.

DISCUSSION

Serum Tg measurement is considered worldwide to be the most sensitive biochemical marker for the post-operative ablation of patients with DTC.[1-6] The numerous assays of serum Tg using polyclonal antibodies are limited by two major problems of variation in

TABLE 1. Comparison of serum Tg contents in commercial internal quality control of C1 and C2 between the one-step standard method and the modified technique using ^{125}I-TgAb 400 µl incubated at 37°C for 90 minutes (n=20).

	Quality control‡	Serum Tg concentrations (ng/ml)		P value
		Gold standard	Modified technique	
Mean (SD)	C1	17.6 (0.60)	18.1 (1.06)	0.097
% CV		3.39	5.83	
Mean (SD)	C2	196.1 (6.30)	193.1 (4.08)	0.112
% CV		3.21	2.11	

‡C1 = low level of serum Tg. C2 = high level of serum Tg.

TABLE 2. Comparison of serum Tg contents in commercial (C1 and C2) and in-house internal quality control sera between the one-step standard method and the modified technique washing the bound form with 2 ml of buffer for 2, 3 and 4 times (n = 20).

Quality control‡	Gold standard†	Modified technique washing with buffer†				
	2 times	2 times	P value	3 times	P value	4 times	P value
Batch 1							
C1	19.21 ± 0.68	19.82 ± 0.88	0.095	-		-	
C2	175.19 ± 3.09	174.23 ± 4.50	0.369	-		-	
Batch 2							
C1	19.21 ± 0.68	-		18.05 ± 0.82	0.062	17.52 ± 0.89	0.043*
C2	175.19 ± 3.09	-		179.54 ± 2.82	0.814	178.63 ± 2.99	0.000*
In-house Low Tg	4.75 ± 0.23	-		4.74 ± 0.33	0.087	4.78 ± 0.38	0.079
In-house Medium Tg	14.33 ± 0.62	-		14.02 ± 0.75	0.362	13.71 ± 0.75	0.066
In-house High Tg	293.38 ± 6.12	-		276.49 ± 5.38	0.000*	275.22 ± 5.24	0.000*

†Data were presented as mean ± standard diviation. *significant at P value < 0.05. ‡C1 = low level of serum Tg. C2 = high level of serum Tg.

TABLE 3. Effect of radioisotope bound counting times of 1, 2, and 3 minutes on serum Tg contents in commercial internal quality control of C1 and C2 (n = 20).

Quality control‡	Counting time (min)	Serum Tg (ng/ml)†		P value
		Gold standard	Modified technique	
C1	1	19.21 ± 0.68	19.80 ± 0.88	0.095
	2	-	19.44 ± 0.80	0.483
	3	-	19.12 ± 0.83	0.793
C2	1	175.19 ± 3.09	174.20 ± 4.50	0.369
	2	-	177.16 ± 3.99	0.415
	3	-	175.53 ± 2.51	0.814

†Data were presented as mean ± standard diviation.
‡C1 = low level of serum Tg. C2 = high level of serum Tg.

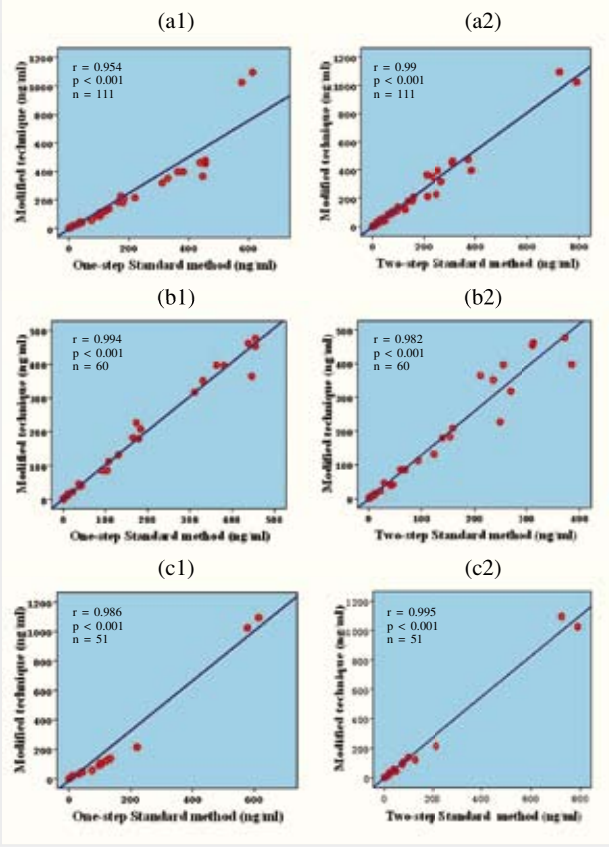

Fig 2. Comparison for serum Tg measurement between the modified technique and the standard method of both IRMA one-step and two-step in 111 DTC patients with both TgAb-negative and TgAb-positive (a1,a2), TgAb-negative (b1,b2) and TgAb-positive (c1,c2).

serum Tg determination and interference from anti-Tg autoantibodies.[5] In radioimmunoassay (RIA), an overestimation result of serum Tg measurement would occur if ^{125}I-Tg bound to endogenous TgAb and prevented it from participating in the competitive reaction. Underestimation would result if the second antibody lacked species specificity and precipitated tracer bound to endogenous TgAb.[1] In immunometric assay (IMA), serum Tg is often undetectable in TgAb-positive DTC patients with disease, TgAb-positive normal euthyroid subjects, and TgAb-positive patients with Graves' thyrotoxicosis.[11]

The presence of heterophilic antibodies (human anti-mouse antibodies, HAMA) in patient sera can form a bridge between the capture and detection antibodies, leading to a false-positive result in the absence or presence of analyte. Very rarely, HAMA can also lead to false-negative or false low results.[9] Assay manufacturers have reduced the incidence of HAMA interferences from the 2-5% observed in unblocked assay by adding blocking reagents, but have been unable to completely eliminate the problem.[9-10] HAMA interference in the commercial kit is protected by adding non-specific mice immunoglobulins tracer.[2,6] The use of monoclonal antibodies for non-competitive assay (IRMA) has been developed by several investigators.[6-14]

The highly specificity test of the present serum Tg measurement technique with analytical sensitivity of 0.28 ng/ml is better than that determined by the standard method and that of Spencer CA et al of 0.6 and 1.0 ng/ml, respectively.[6,13] Our functional sensitivity of less than 1 ng/ml is similar to the Electrochemiluminescent (ECLIA) method of Elecsys 2010[13] as reported by the Elecsys and cobas e analyzers technique. The new technique has potentially greater functional sensitivity

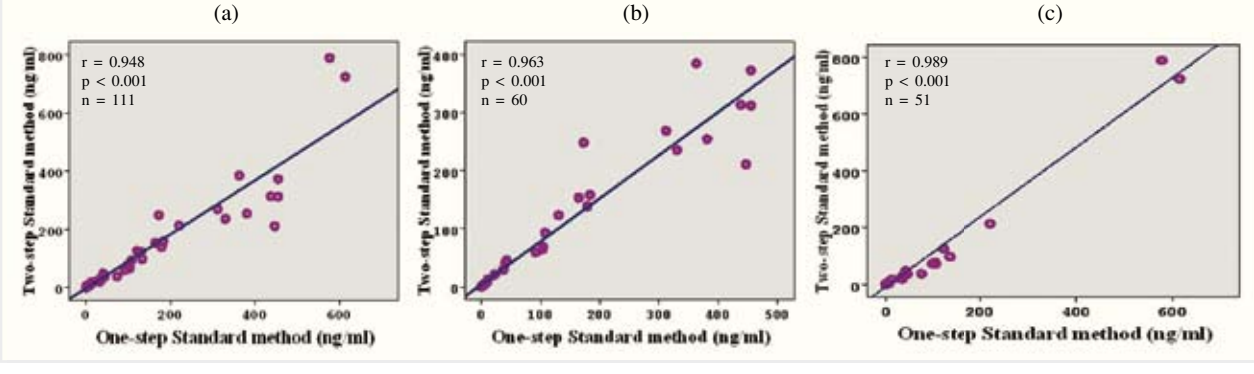

Fig 3. The correlation of the gold standard method between one-step and two-step in the 3 groups of DTC patients with both TgAb-negative and TgAb-positive (a), TgAb-negative (b) and TgAb-positive (c).

than the previous one reported by Okosieme OE, et al., (2 ng/ml).[2] The UK guideline recommends that a detectable serum Tg > 2 ng/ml under TSH stimulation is highly suggestive of residual or recurrent tumor, but could also indicate persistence of a remnant of normal thyroid tissue. The cut-off of 2 ng/ml has been provided by Spencer CA, et al.[13] Our cut-off values of 0.6 and 0.26 ng/ml seum Tg were preliminary studied in 85 DTC patients under TSH stimulation and on T4 suppression. The sensitivity and specificity of off T4 and on T4 were 89.5%, 88.9% and 75.0%, 96.2%.

In conclusion, the two hours TAT of the modified technique for serum Tg measurement shows improved sensitivity and reproducibility when compared with the gold standard method. Moreover, it could be an appropriate marker for follow-up patients with positive and negative TgAb thyroid carcinoma, under either TSH stimulation or suppression therapy.

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REFERENCES

1. Spencer CA, LoPresti JS. Technology insight:measuring thyroglobulin and thyroglobulin autoantibody in patients with differentiated thyroid cancer. Nature Clinical Practice Endocrinol Metab. 2008;4:223-33.
2. Okosieme OE, Evans C, Moss L, Parkers AB, Premawardhana LDKE, Lazarus JH. Thyroglobulin antibodies in serum of patients with differentiated thyroid cancer: Relationship between epitope specificities and thyroglobulin recovery. Clin Chem. 2005 Apr;51(4):729-34.
3. Park EK, Chung JK, Lim IIH, Park DJ, Lee DS, Lee MC, et al. Recurrent/ metastatic thyroid carcinomas false negative for serum thyroglobulin but positive by posttherapy I-131 whole body scans. Eur J Nucl Med Mol Imaging. 2009 Feb;36(2):172-9.
4. Morgenthaler NG, Froehlich J, Rendl J, Willnich M, Alonso C, Bergmann A, et al. Technical evaluation of a new immunoradiometric and a new immunoluminometric assay for thyroglobulin. Clin Chem. 2002 Jul;48(7): 1077-83.
5. Plecheczyk M, Baldet L, Pau B, Bestide JM. Novel immunoradiometric assay of thyroglobulin in serum with use of monoclonal antibodies selected for lack of cross-reactivity with autoantibodies. Clin Chem. 1989 Mar; 35(3):422-4.
6. Sriprapaporn J, Satayaban B, Amornkittcharoen B, Rojvatunyu A, Pattanachak C, Thongpraparn T. Prognostic value of serum thyroglobulin determination after thyroidectomy for differentiated thyroid carcinoma. Intern Med J Thai 2006; 22(1): 20-6.
7. Munro AC, Chapman RS, Templeton JG, Fatori D. Production of primary antisera for radioimmunoassay. In: Hunter WM, Corrie JET eds. Immunoassays for clinical chemistry. Edinburgh: Churchill Livingstone; 1983. p. 447-55.
8. Spencer CA. New insights for using serum thyroglobulin (Tg) measurements for managing patients with differentiated thyroid carcinomas. Thyroid Int. 2003;4:1-14.
9. Preissner CM, Dodge LA, O'Kane DJ, Singh RJ, Grebe SK. Prevalence of heterophilic antibody interference in eight automated tumor marker immunoassays. Clin Chem. 2005 Jan;51(1):208-10.
10. Preissner CM, O'Kane DJ, Singh RJ, Morris JC, Grebe SK. Phantoms in the assay tube: heterophile antibody interferences in serum thyroglobulin assays. J Clin Endocrinol Metab. 2003 Jul;88(7):3069-74.
11. Spencer CA. Challenges of thyroglobulin (Tg) measurement in the presence of Tg autoantibodies (TgAb). J Clin Endocrinol Metab. 2004 Aug; 89(8):3702-4.
12. Morgenthaler NG, Froehlich J, Rendl J, Willnich M, Alonso C, Bergmann A, et al. Technical evaluation of a new immunoradiometric and a new immunoluminometric assay for thyroglobulin. Clin Chem. 2002 Jul;48(7): 1077-83.
13. Spencer CA, Bergoglio LM, Kazarosyan M, Fatemi S, LoPresti JS. Clinical impact of thyroglobulin (Tg) and Tg autoantibody method differences on the management of patients with differentiated thyroid carcinomas. J Clin Endocrinol Metab. 2005 Oct;90(10):5566-75.
14. Schumberger M, Hitzel A, Toubert E, Corone C, Troalen F, Schlageter MH, et al. Comparison of seven serum thyroglobulin assays in the follow-up of papillary and follicular thyroid cancer patients. J Clin Endocrinol Metab. 2007 Jul;92(7):2487-95.