Rapid intraoperative method for the identification of metastatic lymph nodes from thyroid carcinoma

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Abstract

In this study, we established a rapid method for intraoperative identification of thyroid carcinoma metastatic lymph nodes. We developed a rapid method of thyroid globulin (Tg) detection called Tg-POCT based on time-resolved fluorescence immunoassay and validated reagent performance. Thyroid and lymph node tissues with metastatic thyroid cancer were considered as the positive group, and muscle, fiber, fat, and other tissues were considered as the negative control group to verify the above method combined with fine needle puncture. CV of the same batch number was 8.38% and that of different batch numbers was 11.24%. The minimum detection limit was 0.02 ng/mL. The test conformity of tissue samples was 100% by using the self-designed reference value. Thus, Tg-POCT is feasible as a rapid intraoperative method to identify thyroid cancer metastatic lymph nodes.

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Introduction

In recent years, we attempted to determine thyroglobulin (Tg) content by fine needle aspiration (FNA) eluent, which showed positive significance in the qualitative analysis of thyroid tumor-related cervical lymph nodes [1-2]. Tg is a macromolecule glycoprotein, synthesized only by thyroid epithelial cells, with a relative molecular weight of 660 KD, and its presence can indicate the existence of thyroid follicular epithelial cells.

In addition to the high levels content of Tg in normal thyroid tissues, high levels of Tg are observed in well-differentiated thyroid cancer tissues and lymph node tissues with thyroid cancer metastasis [3].Therefore, Tg detection by puncture cell eluent can be used as a supplement to FNA to improve accuracy in identifying the metastatic lymph nodes of thyroid cancer. In addition, the rapid detection of Tg in puncture cell eluent during surgery will significantly improve the quality of thyroid cancer surgery and reduce the risk of postoperative recurrence and metastasis.

In this study, a rapid detection method based on time-resolved fluorescence microsphere immunochromatographic assay of Tg microspheres was established; we also verified its performance and conducted preliminary clinical comparisons.

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Materials and Methods

Data

A pair of antibodies (From Sigma-Aldrich Company, Building C, qiantan world trade center (phase ii), no. 3, lane 227, Dongyu road, Pudong new area, Shanghai, China) was used to capture the Tg present in samples. Goat anti-rabbit immunoglobulin and rabbit IgG (From Sigma-Aldrich Company, Building C, qiantan world trade center (phase ii), no. 3, lane 227, Dongyu road, Pudong new area, Shanghai, China) were used as quality control to ensure the accuracy of test results. Thyroglobulin antigen (From Roche Group, 1100 Longdong Road, Pudong new area, Shanghai, China) was used to substitute tissue in the experiments. Fluorescent microspheres (From Thermo Fisher Scientific, 27 Xinjinqiao road, Pudong new area, Shanghai, China) bind to antibodies and were used for detecting antigen–antibody binding. Analyzing membrane, water absorbing paper, sample pad, and binding pad (From GE,1 Yongchang North Road, Beijing, China) were included in the testing system. The test samples were obtained from the pathological examination of tissue types confirmed by the pathology department. Thyroid and lymph node tissues with metastatic thyroid cancer were included in the positive group, and muscle, fiber, fat, and other tissues were included in the negative control group.

Methods

After ultrasonic resuspension, 1 mL of the fluorescent microspheres were placed in a 1.5 ml EP tube and centrifuged at room temperature for 15 min at a centrifugation speed of 1×104 r/min. Further, 0.02 mol/L 2-(N-morph) ethanesulfonic acid(MES) buffer was used. The pH was washed from 2.0-8.0 twice and the 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) activation solution containing 2 mg/mL was added. After being activated at room temperature in darkness for 30 min, washing with 0.02 mol/L phosphate buffer (PBS), ultra-pure water, 0.05 mol/L HEPES buffer or 0.05 mol/L Tris-HCl buffer was performed twice. The above liquid was added according to the microsphere: the mass of the antibody was 1-20 μg, and the labelled antibody was added and conjugated for 2 h in darkness. After the binding was completed, a sealant equivalent to 1/20 of the volume of the coupling solution was added. The sealant was 0.02 mol/L PBS buffer containing 10% bovine serum albumin (BSA), ultra-pure water, 0.05 mol/L HEPES buffer or 0.05 mol/L Tris-HCl buffer. The buffer system of the sealant was the same as that of the binding

buffer system. The reaction was allowed to proceed for 30 min in darkness. After centrifugation for 15 min, the supernatant was discarded and washed twice with deionized water. Then, the supernatant was redissolved with 0.02 mol/L PBS buffer containing 1% BSA (pH 7.4), and the above liquid was diluted to 1%-10% of the original concentration using 0.02 mol/LPBS buffer containing 1% BSA and 0.1% polysorbate and then stored as sample reaction solution at 4.(Figure1. Labeling of fluorescent microspheres)

NC membranes were laid on the bottom plate of polyvinyl chloride (PVC), and the coated antibody and quality control antibody were diluted to 0.1-1 mg/mL using 0.02 mol/L PBS solution (pH 7.4) containing 1% sucrose. The two were evenly sprayed on the NC film at an interval of 0.5 cm and 1.25 L/cm to form a detection line (T) and quality control line (C). The diluent was sprayed on the binding pad with a quantity of 150 μ L per 1.5×30 cm^2. After 4 h of drying at 30 in the drying box, we laid absorbent paper and sample pads on both sides of the NC film. At 35% humidity, the test strip was cut into 0.4 cm-wide strips at 25 and put into the clamp to form the test strip.(Figure2. The composition of speed measuring card

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The above reagent allowed to warm to room temperature. Tg antigen (approximately 100 ng/mL) was considered to be a high-value sample, and normal saline was considered to be a zero sample. Further, 50 μL of Tg antigen was added to the reagent strip and 50μL of sample reaction solution was added. The ratio of high-value sample/zero sample was defined as signal-to-noise ratio, and the method with the highest signal-to-noise ratio was selected as the optimal scheme.(Figure3. Testing Flow)

Statistical analyses

SPSS 19.0 software was used to analyze various performance indices of Tg-POCT kits and to assess the specificity and sensitivity of clinical tissue samples.

Results

During the research and development process, we found that pH and the amount of antibody had a great impact on the quality of the reagent, so we assessed the effect of the main indicators of each key link in the production process.

Effect of pH of activated liquid

The pH gradient of the microsphere during activation was 2.0 –8.0, and the optimum pH was 4.5.(Figure4. effect of pH on activation)

Effect of the pH of the coupling liquid

The pH gradient of the microsphere in the conjugated antibody was 5.0–12.0, and the optimum pH was 8.0.(Figure5. effect of pH on coupling)

Effect of buffer solution

During microsphere coupling, the buffer systems were PBS buffer, ultrapure water, HEPES buffer, and tris-HCl buffer. PBS was the optimum buffer solution.(Figure6.effect of buffer solution)

Effect of amount of antibody for labelling

The amount of antibody used for labelling was 1—20 μg. The optimum amount of antibody was 10 μg.(Figure7. effect of antibody labelled amount)

Effect of amount of antibody for coating

The amount of antibody used for coating was 1—20 μg; 0.8 μg was the optimum amount of antibody for coating.(Figure8.effect of antibody coated amount)

Effect of dilution ratio of fluorescent microspheres

The dilution ratio gradient of fluorescent microspheres was 1%–10%, and the optimum dilution ratio was 3%.(Figure9.effect of dilution ratio of fluorescent microsphere)

Performance verification

Standard curve

Tg reference calibrators of 10 ng/mL, 50 ng/mL, 100 ng/mL, 200 ng/mL, and 300 ng/mL were added to Tg-POCT test paper as a drop of 60 μL. After reaction at 30 for 5 min, the test was performed. The T/C value, which was the signal value, was measured five times and CV was counted. According to the statistical method, T/C ratio of the detected sample was taken as the ordinate, and the concentration of Tg standard was taken as the X-coordinate. The equation was established, and the standard curve was synthesized. Linear regression coefficient was 0.976. The high value was partly due to the hook effect. Overall, the curve was linear and smooth.(Table1. standard curve Figure10. standard curve)

Table1. standard curve

reference calibrators(ng/mL)	signal value	CVs(%)
10	1.31±0.054	4.12
50	2.79±0.119	4.27
100	4.08±0.162	3.97
200	5.69±0.205	3.60
300	6.81±0.273	4.01

Stability and degree of precision

The 60 μL Tg reference standards (10 ng/mL, 300 ng/mL) were added to the test strip, and the fluorescence values of the quality control line and detection line were read on the immunoassay machine after reaction at 30 for 5 min, and the measurements were repeated 10 times. The reagent was placed at 4 for 6 months and tested again. The test was repeated 10 times. The CVs in 10 ng/mL point test batch were 9.62%, 13.05%, 7.14%, and 9.43% in 300 ng/mL. Considering that the fluctuation of fluorescence values in the low concentrations were higher than those in the high concentrations owing to human error and other reasons, it was normal that the low concentration value CV was higher than the high concentration CV value. In conclusion, the Tg-POCT reagent has good stability and degree of precision.(Table2. stability and degree of precision)

Table2. stability and degree of precision

values (ng/mL)	CVs(%) same batch	CVs(%) different batches
10	9.62	13.05
300	7.14	9.43

Specificity

Specificity

Take the prepared standard product thrombopoietin (TPO) and the corresponding standard product Tg and test paper at the same time. The cross-reaction rate (CR%) at each concentration was calculate as TPO/Tg × 100%. Within the concentration range used in this study, the cross-reaction rate with TPO was less than 0.1%, indicating that there was no cross-reaction.

Minimum detection limit

The 60 μL standard blank points were added onto the test strip. After reaction at 30 for 5 min, the test was

repeated 10 times. The T/C ratio was calculated and the concentration was measured using the standard curve. The mean value of T/C was 0.062, which was converted to 0.02 ng/mL on the standard curve. This value was the sensitivity of the test group.(Table3. Minimum detection limit)

Table3. Minimum detection limit

No.	C Line	T Line	signal value
1	52941	4235	0.08
2	54502	2725	0.05
3	49881	3492	0.07
4	51092	2555	0.05
5	48997	2940	0.06
6	51436	2057	0.04
7	52377	2619	0.05
8	49450	3462	0.07
9	48887	4889	0.1
10	52996	2650	0.05
?X	51255.9	3162.276	0.062

Tissue test results

The samples were tissue samples from the pathology department with definite pathological results. Take 14 cases of pathological results for Tg can normal secretion of thyroid tissue of thyroid carcinoma and 10 cases of pathological results have metastatic lymph node tissue as positive group, 45 cases of pathology confirmed as unable to secrete Tg of thyroid tissue, including 13 cases of fat tissue, muscle tissue in 7 cases, 10 cases of fibrous tissue, lymph node tissue 15 cases (not shift) as a negative control group separately puncture test. The value of $>$ 35ng/mL was positive, the detection rate was calculated, and the concentration was calculated with the 3.1 standard curve. SPSS 19.0 software was used to analyze the concentration values. The paired t-test was used to test the concentration values. $P<0.05$ was considered to be statistically significant. The results were as follows(Table4.Tissue test results)

Table4.Tissue test results

Tissue	1 -2	1 - 3	1- 4	1 - 5	1 - 6	2 - 3	2- 4	2 -5	2- 6
t	1.08	4.94	3.69	3.99	5.03	7.27	6.57	7.27	7.27
P	0.31	0.00	0.01	0.00	0.00	0.00	0.00	0.00	

1.Thyroid; 2.Lymph(with thyroid cancer cells); 3.Fat; 4. Muscle; 5. Fiber; 6.Lymph(normal); 7.positive group; 8.negative control group

Thyroid tissue and metastatic lymph nodes of tissue results difference ($P > 0.05$), and other organizations results than there were significant differences ($P < 0.05$);Had metastatic lymph nodes and other non-thyroid tissue results have significant differences ($P < 0.05$), results are obvious different positive group and negative control group ($P < 0.05$).(Table5.The coincidence rate with pathological results)

Table5.The coincidence rate with pathological results

pathological results	pathological results	FNA-Tg	FNA-Tg
		-	+
positive group	Thyroid	0	14
	Lymph(lesion)	0	10

pathological results	pathological results	FNA-Tg	FNA-Tg
negative control group	Fat	13	0
	Muscle	7	0
	Fiber	10	0
	Lymph(normal)	15	0

The coincidence rate of positive thyroid tissue and negative non-thyroid tissue was 100% using the Tg-POCT test reagent. Tg-POCT test reagent can be used to distinguish tissues that can normally secrete Tg from those that cannot.

Discussion

Thyroid cancer is a common endocrine malignant tumor, accounting for approximately 1% of all malignant tumors [4]. The incidence of thyroid cancer in the Chinese population is 1/300,000–1/200,000, with an annual increase of nearly 5 times since the last 10 years. More than 85% of thyroid cancers are thyroid papillary carcinomas[5]. Lymph node metastasis can occur in the early stage of PTC, but patients can still survive in the long term, provided that treatment is prompt and accurate. For patients with cervical lymph node metastasis, total thyroidectomy and regional lymph node dissection are generally required, after which thyroxine is administered, and I^{131}nuclide ablation is required for high-risk patients. Generally, 5%-20% in situ or local recurrence and 10%-15% distal metastasis will occur after surgery[6-8].Therefore, accurate preoperative identification of the nature of thyroid mass and cervical lymph nodes is crucial for the selection of reasonable surgical methods and treatment programs; in addition, rapid intraoperative identification of the nature of cervical metastatic lymph nodes is particularly important for favorable prognosis of thyroid tumor patients.

At present, the diagnostic methods for suspected cervical metastatic lymph nodes of thyroid cancer mainly include high-frequency ultrasound (B ultrasound), neck CT, fine needle aspiration biopsy cytology (FNAC), and other examination methods. Among them, b-mode ultrasound is a convenient and harmless examination method, which can assist in the identification of benign and malignant thyroid nodules, but its main disadvantages are that it cannot reflect the histological characteristics of nodules and that b-mode ultrasound examination is relies heavily on the experience of the examiner, thus being subjective to some extent. The diagnostic value of neck CT for thyroid nodule lesions is not well reported in the literature and is rarely used in the clinical setting. B-ultrasound guided FNAC is currently recognized as the most direct method to identify the nature of the mass and lymph node. It has been reported that the sensitivity and specificity can reach 87% and 76%, respectively, but 20%–40% of puncture specimens cannot be accurately identified, and cytological examination results are quite influenced by the experience of the technician[9-10]. The above diagnostic methods are mainly used for preoperative identification and prognosis, but these cannot be used intraoperatively due to the need for large instruments or the prolonged examination time.

Traditional methods for Tg detection mainly include radioimmunity, enzyme-linked immunoassay, and chemiluminescent immunoassay, but these methods require large instruments and require a long detection time, which are not suitable for rapid intraoperative detection. Fluorescence microsphere immunochromatography is a novel, innovative quantitative detection technology developed by combining time-resolved fluorescence immunoassay and traditional immunochromatography. It has the characteristics of high sensitivity, good stability [11], and short detection time.

Conclusions

In this study, a rapid intraoperative detection method for Tg was developed, which is characterized by good stability, high sensitivity, and high specificity. Combined with intraoperative fine needle puncture, it can quickly help to detect thyroid cancer metastasis in the lymph nodes, which will help to improve the efficiency and quality of surgical treatment.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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Figure1. Labeling of fluorescent microspheres

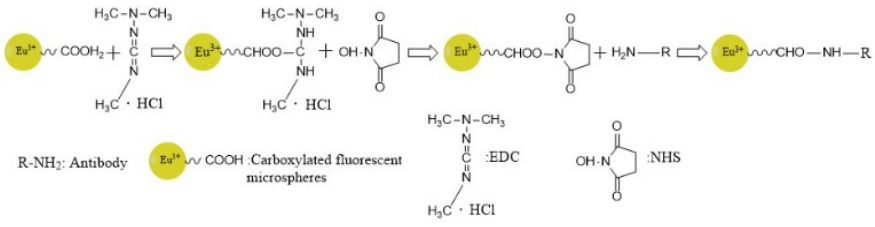
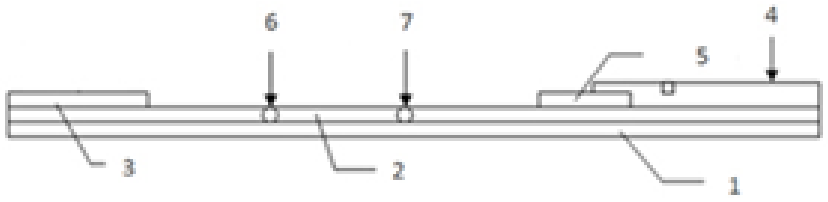


Figure2. The composition of speed measuring card



1.plastic back plate 2.analyzing membrane 3.water absorbing paper 4.sample pad 5.binding pad 6.control line 7.test line

Figure3. Testing Flow

Figure4. effect of pH on activation

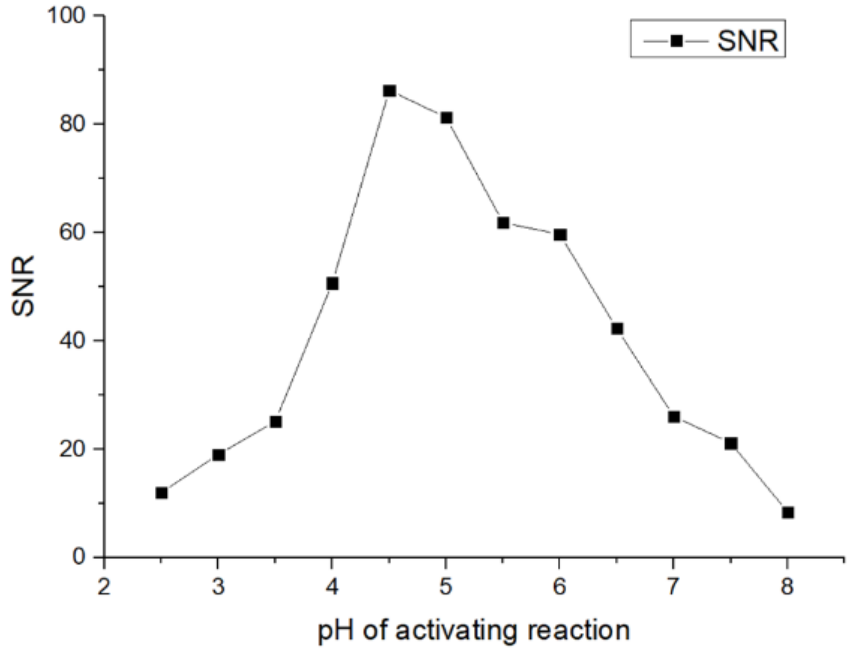


Figure5. effect of pH on coupling

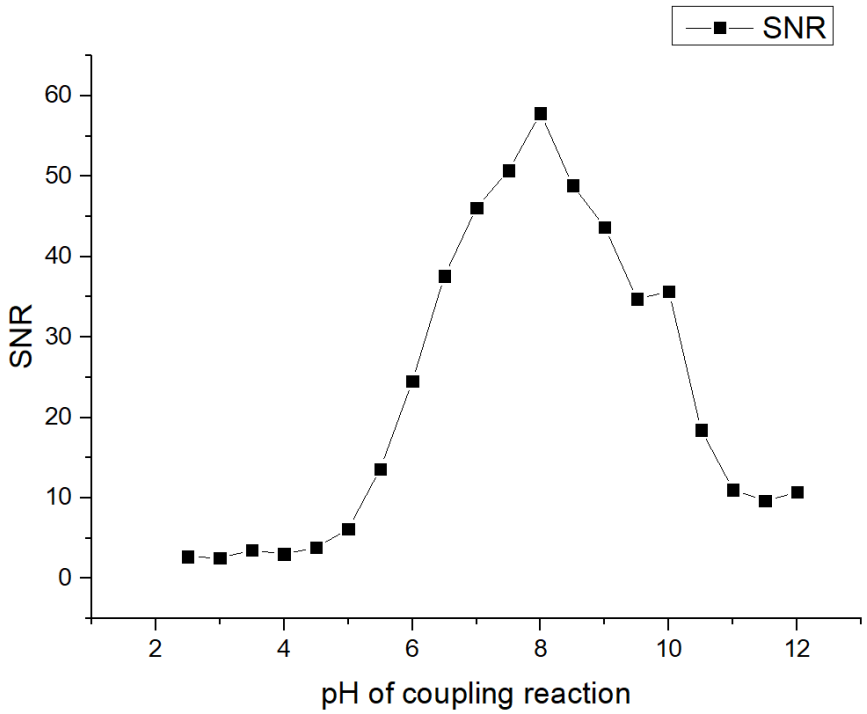


Figure6.effect of buffer solution

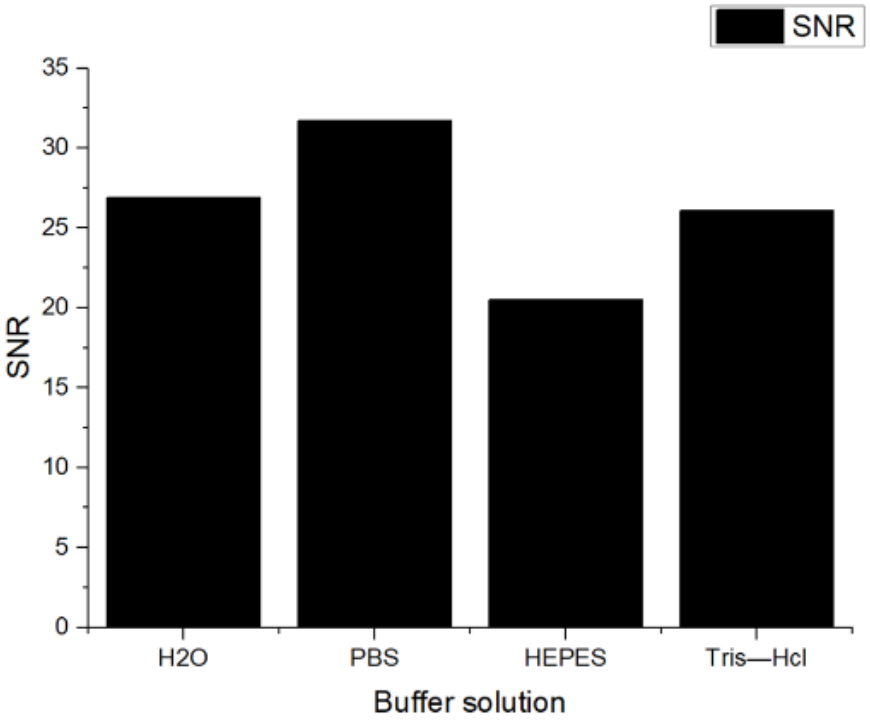


Figure7. effect of antibody labelled amount

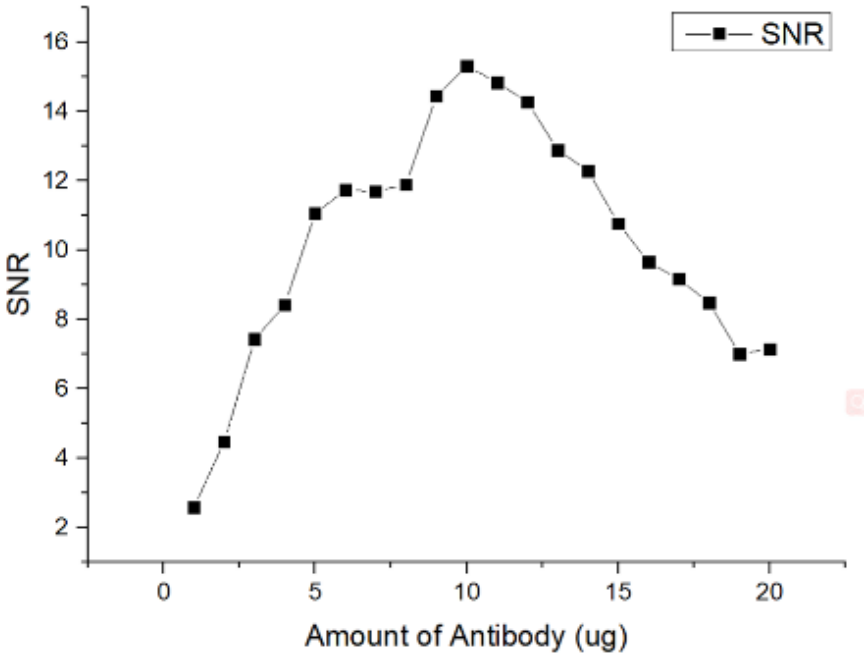


Figure8.effect of antibody coated amount

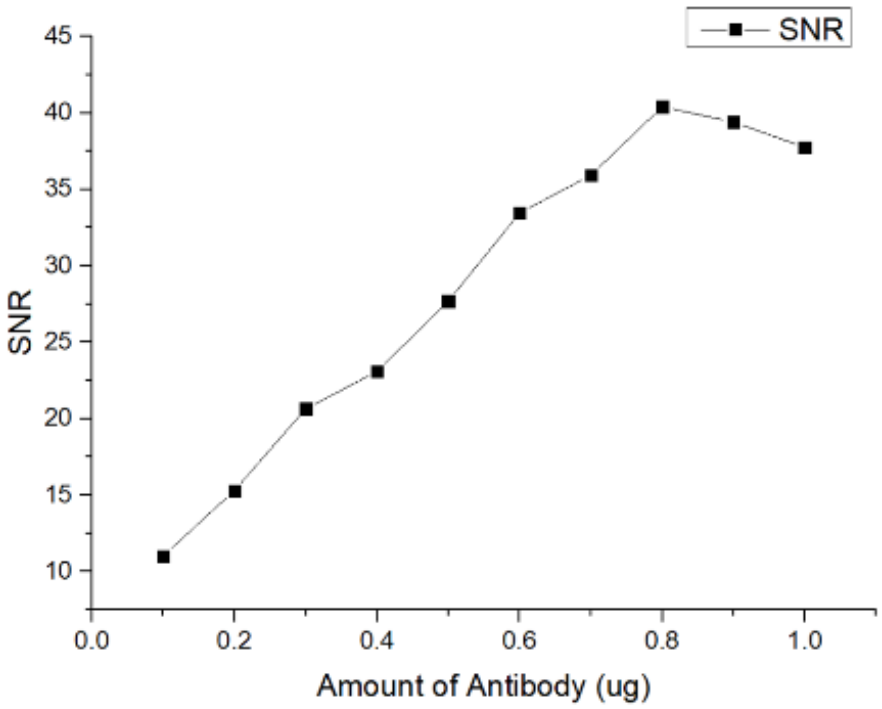


Figure9.effect of dilution ratio of fluorescent microsphere

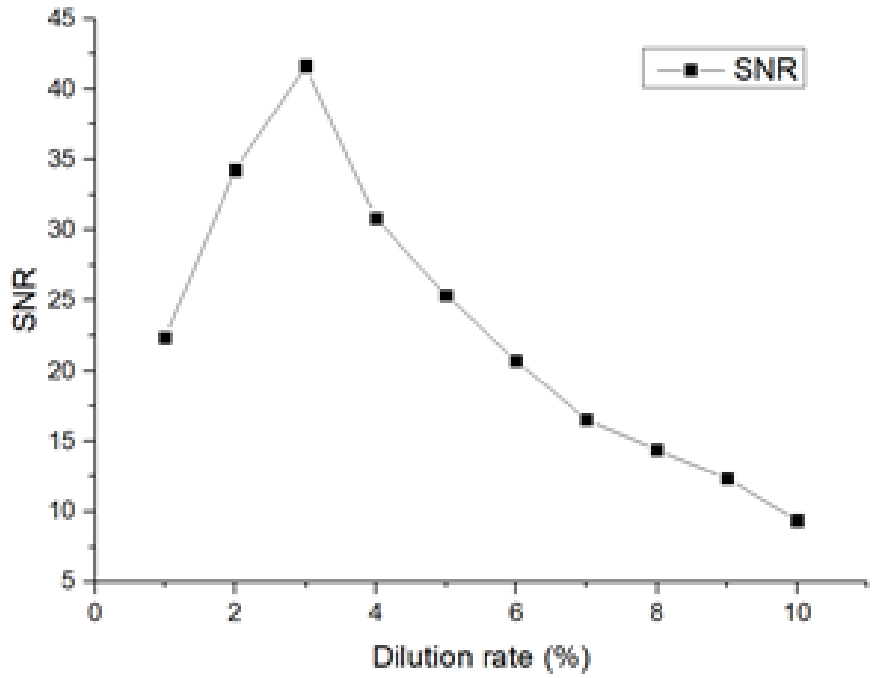
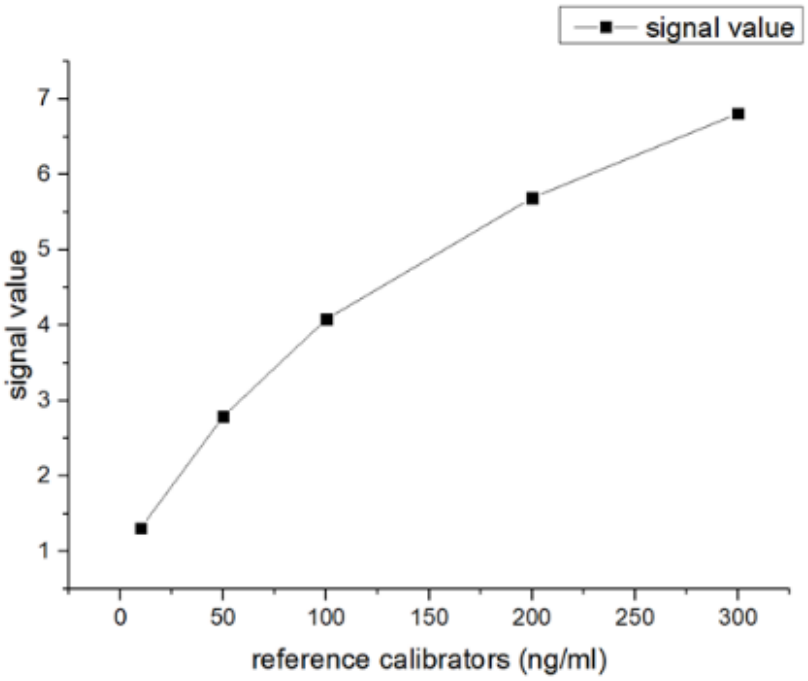


Figure10. standard curve



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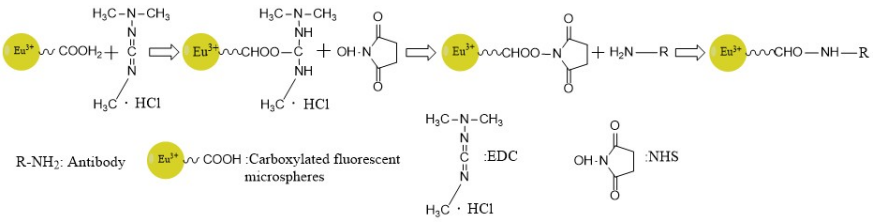

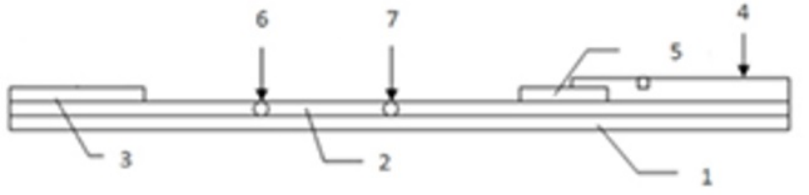

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