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RESEARCH ARTICLE

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Enhanced chemiluminescence enzyme-linked immunoassay for the determination of DNA methyltransferase 1 in human serum

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Abstract

The occurrence of many diseases is closely related to the high expression of DNA methyltransferase 1 (DNMT1). However, most studies are focused on the detection of DNMT1 activity, a few are concerned with the detection of DNMT1 content. In this study, we developed a simple and highly sensitive chemiluminescence (CL) assay for the detection of DNMT1 content. In this method, anti-DNMT1 monoclonal antibody was coated on a polystyrene microplate to capture DNMT1. Then anti-DNMT1 polyclonal antibody and goat anti-rabbit immunoglobulin G with horseradish peroxidase (IgG-HRP) were respectively added to combine with captured DNMT1 to form a sandwich structure. Finally, the HRP could catalyze CL substrate and achieve CL signal response. Based on this novel sensitive strategy, the recovery percents were in the ranges from 71.5% to 91.0%. The precision of intra-assays and inter-assays were 5.45%–11.29% and 7.03%–11.25%, respectively. The method was successfully applied for the determination of DNMT1 in human serum. The detection results of serum samples showed that the proposed assay had a high correlation with enzyme-linked immunosorbent assay (ELISA) kit. Compared with the ELISA kit (limit of detection = 0.1 ng/mL), the method has a lower limit of detection of 0.042 ng/mL. Therefore, our method has the potential for the detection of DNMT1 content in clinical diagnosis.

KEYWORDS

antibody, chemiluminescence, content, DNA methyltransferase 1

1 | INTRODUCTION

DNA methylation, a common epigenetic modification, occurs at the C-5 position of the pyrimidine ring of cytosine within CpG sites and at the N-6 position of the purine ring of adenine in tetranucleotides. It plays a critical role in gene transcription, cellular differentiation, and genomic stability.[1] Aberrant DNA methylation has the ability to deactivate the tumor suppressor genes and has been closely related to many types of genetic disorders and human malignancies.[2] DNA methylation is catalyzed by DNA methyltransferases (DNMTs) using S-adenosylmethionine as the methyl donor.[3,4] Previous research has identified three different types of DNMTs in mammals, namely DNMT1, DNMT2 and the DNMT3 family, the DNMT3 family includes DNMT3a, DNMT3b and DNMT3L.[5] Among them, DNMT1 is essential for catalyzing and sustaining DNA methylation, and is an independent prognostic factor in clinical diagnosis.[6] A new paper has shown that DNMT1 can be used as initial DNMTs.[7] Many studies have shown that DNMT1 has a profound effect on severe cancers such as lung cancer,[8] breast cancer,[9,10] gastric cancer,[11] cervical cancer,[12] and colon cancer.[13,14] As a promising tumor biomarker, DNMT1 has received great attention in molecular biology,[15] biosensor,[16–18] preventive medicine,[19] and clinical research.[20,21]

Many methods have been developed for the detection of DNMT1, including electrochemical assay,[22,23] high-performance liquid chromatography,[24] fluorescence,[25,26] and colorimetry.[27] However, most of the earlier mentioned studies concentrate on the detection of DNMT1 activity, only a few focus on the detection of DNMT1 content. Actually, to some extent, the detection of DNMT1 activity *in vitro* is not accurate because DNMTs may have been inactivated after extraction or treatment. Therefore, compared with DNMT1 activity, DNMT1 content may better reflect DNA methylation level. Besides, there are some shortcomings in the earlier methods. For example, electrochemistry assay often requires complicated laborious pretreatment. High-performance liquid chromatography requires sophisticated instruments leading to high cost. The high background and low sensitivity of fluorescence and colorimetry methods restrict their further development and application. In addition, these methods are confined to laboratory studies and have not been applied to detect DNMT1 levels in serum samples. Thus, high-throughput screening cannot be achieved in early clinical diagnosis.

Chemiluminescence (CL)[28] has been widely used in various fields such as immunology, analytical chemistry and molecular biology[29,30] because of its advantages of high sensitivity, wide dynamic range and low background interference. Recently, several related studies have reported the mechanism and optics application of novel enhanced CL systems.[31,32] In order to improve the detection method of DNMT1, and more accurately reflect the true level of DNMT1 in human serum, we proposed a novel enhanced chemiluminescence enzyme-linked immunoassay (ECLEIA) for the detection of DNMT1 content in real human samples. In the assay, the specific binding of antigen–antibody greatly improved the selectivity of the reaction. Anti-DNMT1 monoclonal antibody (McAb) was immobilized on a polystyrene microplate to achieve rapid separation of the solution, which also provided a basis for the detection of a large number of actual samples. The enzyme-catalyzed process was amplified by adding 4-biphenylol (BIP) as an efficient enhancer to luminol–hydrogen peroxide (H_2O_2)–horseradish peroxidase (HRP) CL system, further reducing the limit of detection.

2 | EXPERIMENTAL

2.1 | Materials

Human DNMT1 was purchased from Sigma Company (St Louis, MO, USA). Anti-DNMT1 McAb and polyclonal antibody (PcAb) were acquired from Biodragon Immunotechnologies Co., Ltd (Beijing, China). DNMT1 enzyme-linked immunosorbent assay (ELISA) kit was purchased from Yunclone Co., Ltd (Wuhan, China). DNMT3a and DNMT3b ELISA kits were bought from Kang Lang Biological Technology Co., Ltd (Shanghai, China). Goat anti-rabbit immunoglobulin G with horseradish peroxidase (IgG-HRP), bovine serum albumin (BSA) were obtained from Solarbio Life Sciences (Beijing, China). All reagents were of analytical grade. The water used in the experiments was deionized and ultrafiltered using a Milli Q system (Waters, Milford, MA, USA).

The following buffers were used: (A) 0.01 mol/L phosphate buffered saline (PBS), pH 7.4; (B) 0.01 mol/L Tris(hydroxymethyl)amino methane–hydrochloric acid buffer (Tris–HCl), pH 8.5; (C) 0.01 mol/L Tris–HCl, pH 10.2; (D) 0.05 mol/L carbonate buffer (CB), pH 9.6; (E) washing buffer, buffer A with 0.05% (*v*/*v*) Tween-20 (PBST), (F) blocking buffer, buffer A with 0.5% (*w*/*v*) BSA.

2.2 | Apparatus

Microplate Reader (Tecan Sunrise, Tecan Group Ltd, Männedorf, Switzerland). CL immunoassay analyzer (Centro xs3 LB960, Berthold Technologies, Oak Ridge, TN, USA). The 96-well polystyrene microtiter plates used in the assay were purchased from Jincanhua Industrial Co., Ltd (Shenzhen, China). The electrical thermostatic cultivation cabinet was from DHG-9146A Jing Hong Laboratory Instrument Co. Ltd (Shanghai, China).

2.3 | Immunoassay procedure

A convenient and sensitive ECLEIA was applied to detect DNMT1 content in human serum. Anti-DNMT1 McAb was coated on a polystyrene microplate to capture the target DNMT1. Subsequently, anti-DNMT1 PcAb was added into the reaction system specifically to bind with the target protein, forming a sandwich immunocomplex. Finally, the concentration of DNMT1 was determined by the luminol–H_2O_2–HRP–BIP CL system. The CL intensity increased with the increase of DNMT1 concentration.

The detailed procedures are shown in Figure 1. A 96-well polystyrene microtiter plate was coated with 100 μL/well of anti-DNMT1 McAb and incubated at 4°C for approximately 12 h. Then, excessive anti-DNMT1 McAb was removed. To reduce non-specific adsorptions, the free binding sites were blocked by PBS with 0.5% (*w*/*v*) BSA. Subsequently, 100 μL/well of DNMT1 standard solutions or serum sample were added onto the microplate, and the mixture was incubated at 37°C for 90 min. Next the wells were washed three times by PBST. Anti-DNMT1 PcAb was diluted 50-fold with blocking buffer, and 100 μL/well of this diluted solution was added onto the same microplate and then again incubated at 37°C for 90 min. After washing, goat anti-rabbit IgG-HRP was diluted with 5000-fold blocking buffer, and 100 μL/well of this diluted solution was added onto the microplate and incubated at 37°C for another 60 min. Lastly, luminol and H_2O_2 was added onto the microplate after washing, and the CL signal in each well was recorded using the CL immunoassay analyzer.

3 | RESULTS AND DISCUSSION

3.1 | Optimization of CL reaction parameters

3.1.1 | The type of buffer for coating antibody

The buffer of coating antibody affects the CL intensity and the background value. Here, four different reagents were considered as buffer

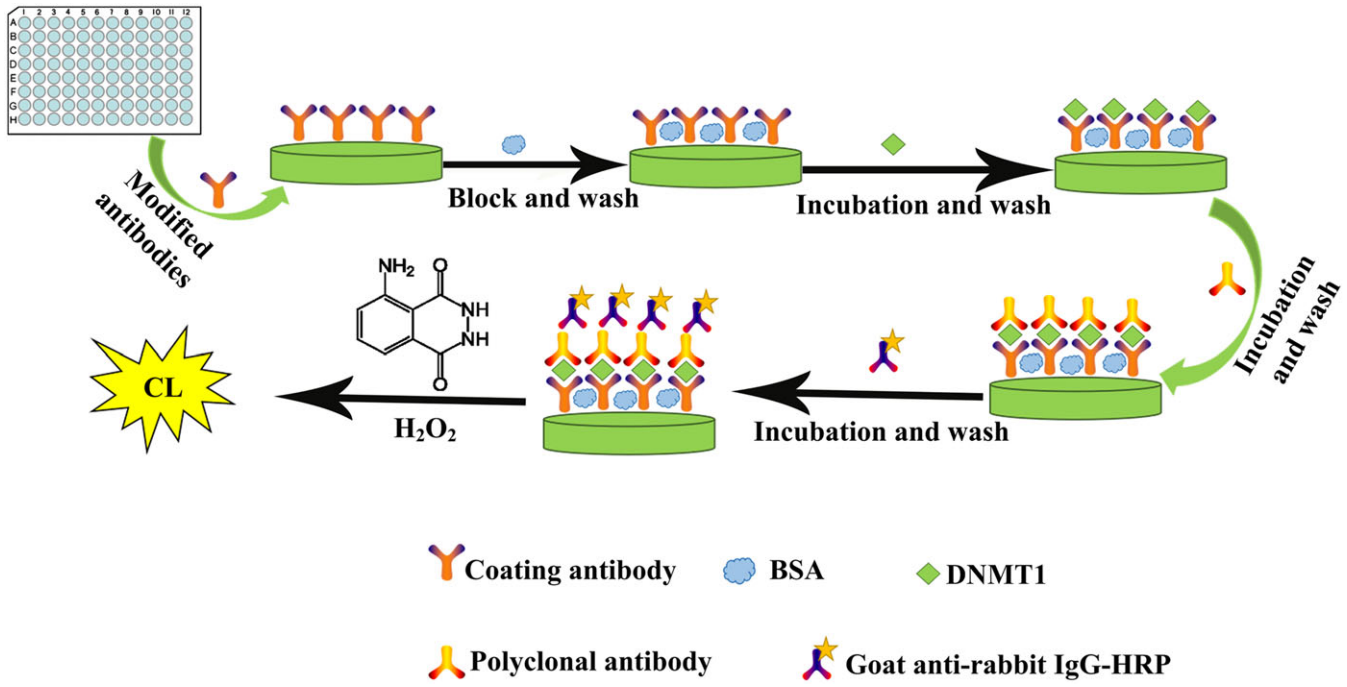

FIGURE 1 Schematic depiction of the detection of DNA methyltransferase 1 (DNMT1) based on enhanced chemiluminescence enzyme-linked immunoassay (ECLEIA)

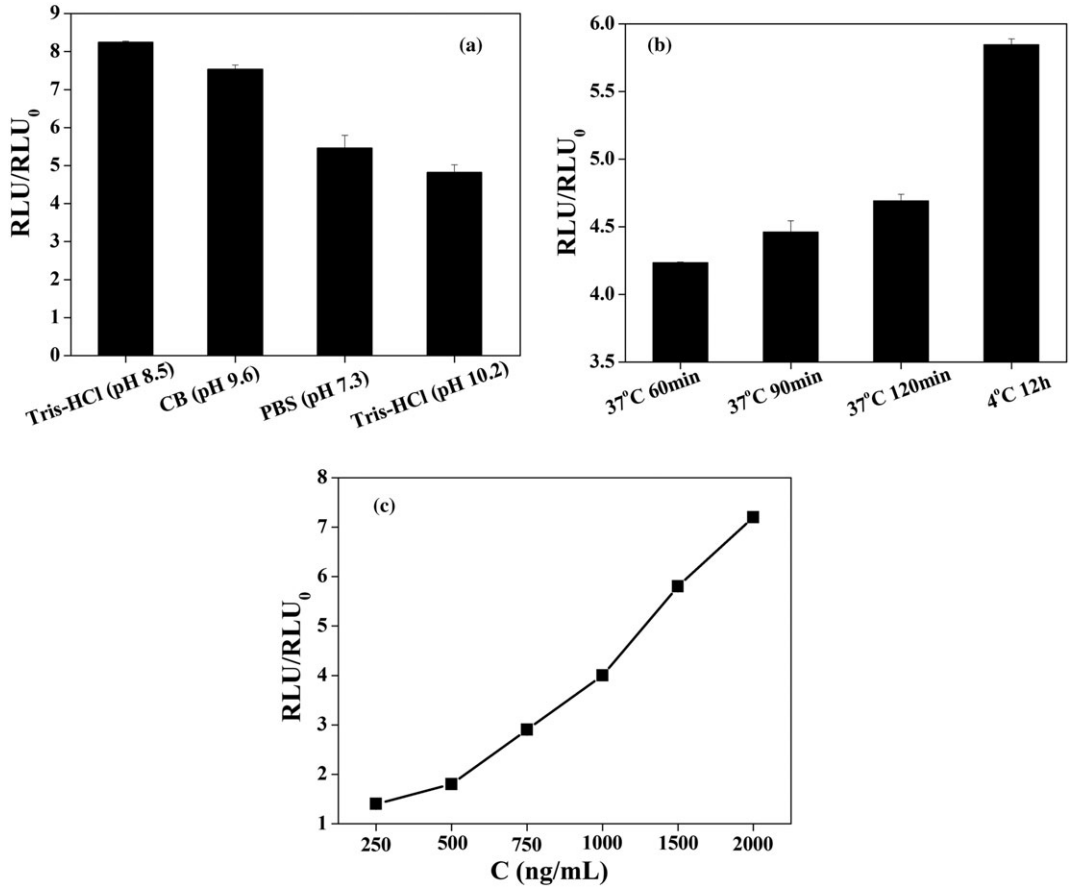

FIGURE 2 Single factor method optimization of the type of buffer (a), reaction time (b) and concentration (C) (c) for anti-DNA methyltransferase 1 (DNMT1) monoclonal antibody (McAb) coating.

of anti-DNMT1 McAb, including Tris–HCl solution (0.01 mol/L, pH 8.5), CB solution (0.05 mol/L, pH 9.6), PBS solution (0.01 mol/L, pH 7.4), and Tris–HCl solution (0.01 mol/L, pH 10.2). The results are shown in Figure 2(a). The RLU/RLU_0 value reached a maximum when the buffer was Tris–HCl (0.01 mol/L, pH 8.5). Therefore, Tris–HCl (0.01 mol/L, pH 8.5) was selected as the optimal reaction buffer for anti-DNMT1 McAb coating.

3.1.2 | Reaction time for coating antibody

Using the Tris–HCl buffer, the optimal reaction time was examined for the antibodies. Four different time groups were set, which were 12 h at 4°C, 120 min at 37°C, 90 min at 37°C and 60 min at 37°C. The effect of time is presented in Figure 2(b). It was observed that the maximal RLU/RLU_0 was achieved at 4°C for 12 h. Hence, 12 h at 4°C was selected as the experimental conditions for coating antibody.

3.1.3 | The concentration of anti-DNMT1 McAb

The concentration of anti-DNMT1 McAb is crucial to the experiment. Lower antibody concentrations may result in weaker CL intensity, and higher antibody concentrations may reduce the sensitivity of the method. Figure 2(c) shows the effect of concentration of anti-DNMT1 McAb. There was a gradually rising CL intensity with the increase of antibody concentration. When the concentration of anti-DNMT1 McAb reached 2000 ng/mL, the maximum RLU/RLU_0 value was attained. However, due to the high costs and sensitivity needs, the anti-DNMT1 McAb concentration of 1500 ng/mL was used in the subsequent experiments.

3.1.4 | The dilution ratio of anti-DNMT1 PcAb and goat anti-rabbit IgG-HRP

The dilution ratio was investigated because the concentrations of anti-DNMT1 PcAb and goat anti-rabbit IgG-HRP were vital factors for CL signal and the sensitivity of the ECLEIA. The results are shown in Figure 3(a). The RLU/RLU_0 value increased with the increase of anti-DNMT1 PcAb concentration. When the dilution ratio was 1:50, the value of RLU/RLU_0 reached a maximum. Hence, the subsequent experiments were conducted using a dilution ratio of 1:50 of anti-DNMT1 PcAb. In Figure 3(b), the effect of dilution ratio of goat anti-rabbit IgG-HRP on CL intensity is shown. It was seen that the value of RLU/RLU_0 increased rapidly initially and then more slowly beyond the 1:5000 dilution ratio. Because the increase of CL intensity was not apparent at a dilution ratio of 1:3000, the dilution ratio of 1:5000 was used in this study.

3.1.5 | The incubation time of anti-DNMT1 PcAb and goat anti-rabbit IgG-HRP

In this experiment, the reaction time of anti-DNMT1 PcAb and goat anti-rabbit IgG-HRP was optimized at 37°C and CL signal was

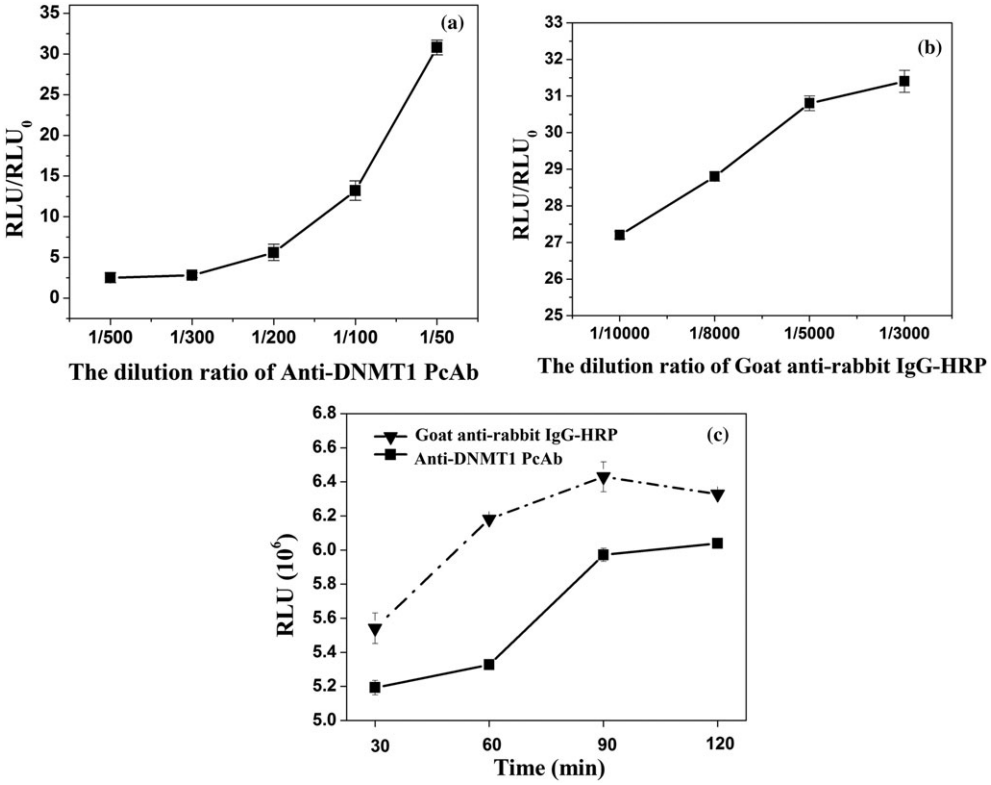

FIGURE 3 Single factor method optimization of anti-DNA methyltransferase 1 (DNMT1) polyclonal antibody (PcAb) dilution ratio (a), goat anti-rabbit IgG-HRP dilution ratio (b), and incubation time of anti-DNMT1 PcAb and goat anti-rabbit immunoglobulin G with horseradish peroxidase (IgG-HRP) (c)

detected under the conditions of 30, 60, 90 and 120 min. The results are presented in Figure 3(c). There was a large number of free antigens in the system at the beginning of the reaction, so the CL signal of anti-DNMT1 PcAb was obviously enhanced within 30 to 90 min. After 90 min, the immune reaction was almost saturated and the CL intensity curve gradually stabilized. Therefore, 90 min was selected as the incubation time of anti-DNMT1 PcAb. For goat anti-rabbit IgG-HRP, the CL signal was enhanced from 30 to 60 min and slowly enhanced within 60 to 90 min. Therefore, the experimental reaction time of 60 min was chosen in the following experiments.

3.2 | Method evaluation

3.2.1 | Calibration curve

Under the conditions of the optimum, ECLEIA was applied to detect DNMT1 content. A calibration curve was obtained by plotting RLU against different concentrations of DNMT1 solutions ranging from 0 to 1500 ng/mL (Figure 4(a)). It was seen that CL intensity increased with the increase of DNMT1 concentration, which was expressed by the estimated linear regression equation [$y = 0.0417x + 6.1788$, $R^2 = 0.9941$, y and x denote the Log_{10}(RLU) and $Log_{10}(C_{DNMT1})$]. Compared with the ELISA kit, the method has a lower limit of detection of 0.042 ng/mL and wider linear range (Table 1).

3.2.2 | Cross-reactivity studies

The specificity of the ECLEIA method was evaluated using DNMT3a and DNMT3b as interferential antigens. The concentrations of all three antigens were 20 ng/mL. The results are presented in Figure 4 (b). Clearly, anti-DNMT1 McAb could easily recognize DNMT1 antigens in the mixture. Even in the presence of DNMT3a and DNMT3b, high CL signal could be obtained and the ECLEIA was not affected by DNMT1 detection.

3.2.3 | Recovery and precision

We evaluated the recovery of DNMT1 by the proposed method. The different amounts of DNMT1 (20, 500, and 1000 ng/mL) were spiked into the mixed normal human serum, and all samples were analyzed three times by ECLEIA. The results are listed in Table 2. The recovery percents were from 71.5% to 91.0%. The relative standard deviations ranged from 5.2% to 10.3%. The results showed that the reliability of ECLEIA was acceptable.

Precision is a key indicator in quantitative analysis, which is closely related to the assay repeatability and accuracy. To evaluate the precision of the immunoassay system, three different concentrations (10, 100, and 1000 ng/mL) of DNMT1 standard solutions were analyzed using the proposed method. The intra-assay precision was obtained by detecting each concentration six times on the same microplate. Similarly, the CL signal was determined in different microplates to

TABLE 1 Comparison of enzyme-linked immunosorbent assay (ELISA) method and enhanced chemiluminescence enzyme-linked immunoassay (ECLEIA) method

Method	Limit of detection (ng/mL)	Linear range (ng/mL)	Linear regression equation	Correlation coefficient
ELISA	0.1	0.5–100	$y = 0.50143x + 1.76886$	$R^2 = 0.9839$
ECLEIA	0.042	0.1–1500	$y = 0.0417x + 6.1788$	$R^2 = 0.9941$

TABLE 2 Recovery of DNA methyltransferase 1 (DNMT1) in serum samples with the proposed enhanced chemiluminescence enzyme-linked immunoassay (ECLEIA) ($n = 3$)

Background (ng/mL)	Added (ng/mL)	Measured (ng/mL)	Recovery (%)	Relative standard deviation (%)
0.6214	20	14.93	71.5	10.3
0.7860	500	432.53	86.3	8.4
0.6495	1000	910.16	91.0	5.6

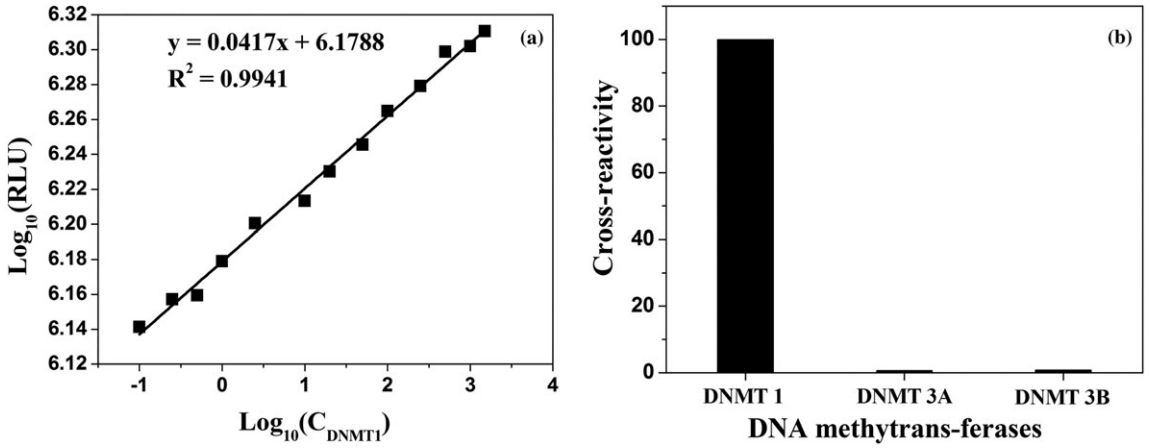

FIGURE 4 Calibration curves for the quantification of DNA methyltransferase 1 (DNMT1) (a), cross-reactivity of the proposed enhanced chemiluminescence enzyme-linked immunoassay (ECLEIA) toward three DNA methyltransferases (DNMTs) (b)

TABLE 3 Precision of the proposed enhanced chemiluminescence enzyme-linked immunoassay (ECLEIA) for DNMT1 detection (n = 6)

Concentration (ng/mL)	Intra-assay			Inter-assay		
	$\bar{X}$ (ng/mL)	Standard deviation	Relative standard deviation (%)	$\bar{X}$ (ng/mL)	Standard deviation	Relative standard deviation (%)
10	10.6	0.93	8.8	9.2	0.73	7.9
100	116.2	4.0	3.4	134.7	3.4	9.4
1000	1034	86.7	8.4	994.7	96.3	9.7

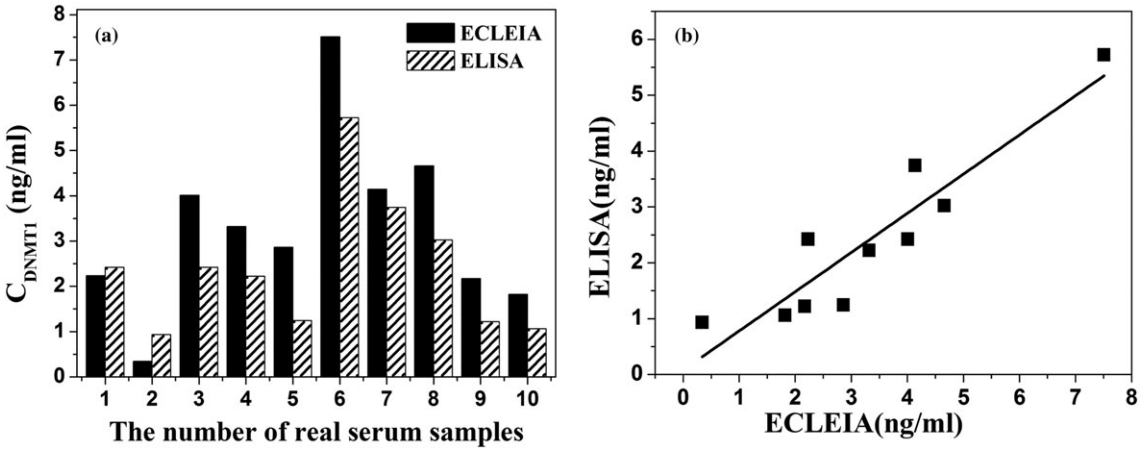

FIGURE 5 Correlation analysis of serum samples results obtained by enhanced chemiluminescence enzyme-linked immunoassay (ECLEIA) and enzyme-linked immunosorbent assay (ELISA) (n = 10)

evaluate the inter-assay precision. The results are presented in Table 3. The precision of intra-assays and inter-assays ranged from 3.4% to 8.8% and 7.9% to 9.7%, respectively. These results showed that the precision of the proposed method was acceptable.

3.3 | Real sample analysis

To demonstrate the potential application of the proposed ECLEIA in clinical research, 10 human serum samples donated by a local hospital (the First Affiliated Hospital of Zhengzhou University, China) were determined by the proposed method and were compared with the widely used ELISA. As shown in Figure 5, all the results of CL detection were confirmed by the ELISA kit. In addition, to further verify the accuracy of the method, paired sample t-test was used to analyze the experimental results. Furthermore, the results (Figure 5) indicated that there was a good correlation between ECLEIA and commercial ELISA kit (correlation coefficient r = 0.922, P < 0.001). Therefore, the proposed method could be applied to detect DNMT1 content in serum.

4 | CONCLUSIONS

Herein, a simple and sensitive ECLEIA was developed for the detection of DNMT1 content. The proposed method showed high precision and satisfied recovery. The test results of actual samples were consistent with the ELISA kit, indicating that the assay has high accuracy. Due to the high sensitivity of CL and the good specificity of antigens and antibodies, the proposed method had a wider linear range and lower limit of detection than the ELISA kit, which can meet requirements for clinical sample detection. Moreover, the experiment was carried out in the 96-well plate, which means multiple samples can be analyzed within a few hours. However, there are still several points that need further improvement of this approach. For example, using magnetic nanoparticles as solid-phase carriers to further improve sensitivity, or using aptamers instead of antibodies to save reaction time. Overall, the proposed approach enables high-throughput screening and has potential application in early diagnosis of cancer.

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