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Establishment of a new protein C detection system based on chromogenic substrate assay and its clinical diagnostic value for deep vein thrombosis

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Abstract

Background: Deficiency of protein C (PC) affects the balance between blood coagulation and fibrinolysis in the human body. Chromogenic-based assay is recommended as the preferred screening method for detecting PC deficiency. We established a PC detection system based on the chromogenic substrate assay.

Methods: First, a kit for the determination of PC activity in plasma was elaborately developed and its reaction parameters on XL-3200c were explored. Then, we evaluated its performance and collected specimens to compare the test results obtained with those of the Siemens detection system. Finally, the clinical diagnostic efficacy of this detection system for deep vein thrombosis (DVT) was assessed.

Results: Optimum conditions for PC detection were 0.25–0.1 U/ml protein C activator Protac® and 2.5–1 mM Pefachrome®PCa5297. The composition and concentration ranges of buffer substances and stabilizers in the kit were also explored. Satisfactory results were observed in performance evaluation. The test results of the newly built detection system were highly correlated with those of the Siemens detection system ($R^2 = 0.9771$ in the control group and $R^2 = 0.9776$ in the DVT group), and Bland-Altman plots also showed high consistency between the two detection systems. In addition, the area under the curve (AUC) of the newly built PC detection system for DVT was 0.888, indicating this system could effectively improve the diagnostic sensitivity and specificity for DVT.

Conclusion: In this study, a sensitive, wide linear range and reliable PC activity detection system were established.

KEYWORDS
chromogenic-based assay, deep venous thrombosis, method comparison, performance evaluation, protein C

1 | INTRODUCTION

Protein C (PC) is a vitamin K-dependent plasma serine protease, with a relative molecular weight of about 62,000 KD, a plasma concentration of 3–5 µg/ml, and a half-life of 6–8 hours.[1] PC exists in the form of a zymogen and has no active biological role. Only when PC is converted to activated protein C (APC) can it perform its anticoagulant activity. The activation of PC is initiated by the

production of thrombin and the formation of a complex with thrombomodulin (TM) on the surface of endothelial cells.[2] Deficiency of this natural anticoagulant may contribute to the weakening of the proteolytic effect of APC on FVa and FVIIIa, thereby resulting in recurrent venous thrombosis and pulmonary embolism.[3]

Laboratory tests for PC currently consist of antigenic assays and functional assays. Compared with the antigenic assays, the advantage of functional assays is that they can evaluate the real biological activity of PC.[4] Functional assays for PC include clotting-based and chromogenic-based assays; the principle of both assays is based on PC being activated to APC to develop its anticoagulant activity. Due to the peculiar feature of the coagulation method being easily affected by a variety of interference factors (such as lupus anticoagulants, heparin, direct thrombin inhibitors), it is now rarely used as a screening test in clinical laboratories.[5] The chromogenic-based assay is used to assess the activity of PC by measuring the change in absorbance; the assay has favorable stability and accuracy and is not easily affected by direct oral anticoagulants (DOACs) and certain interfering enzymes.[6]

In order to further increase the efficiency of PC activity testing, we established a new PC detection system based on the chromogenic assay, conducted preliminary evaluations of performance, and assessed its clinical diagnostic capability to detect DVT.

2 | MATERIALS AND METHODS

2.1 | Specimens

The study was conducted in accordance with the guidelines set by the Ethics Committee of the Affiliated Hospital of Qingdao University. Specimens of 68 DVT patients and 80 healthy people were used for methodological comparison; patients were recruited from the Affiliated Hospital of Qingdao University between November 2020 and June 2021. DVT patients included in this study should be diagnosed for the first time and had not been treated with warfarin, heparin, or DOACs. Normal mixed plasma used for the precision test was mixed with the plasma of apparently healthy subjects (n = 25). Samples from healthy participants (n = 152), who showed normal results on physical examination and routine coagulation tests (activated partial thromboplastin time, prothrombin time, thrombin time, fibrinogen, and D-dimer), were collected to establish the reference interval. All samples were treated with 0.109 mmol/l sodium citrate 1:9 as anticoagulant. Plasma was separated after centrifugation at 1,856 g for 15 min and stored at −80°C until further use. All information about samples was anonymized until the end of detection.

2.2 | Instruments and reagents

2.2.1 | Instruments

(i) XL-3200c (Automated Blood Coagulation Analyzer; ZONCI, Beijing, China) and (ii) CN-6000 (Automated Blood Coagulation Analyzer, Sysmex, Kobe, Japan).

2.2.2 | Reagents

(i) Raw materials needed for the new PC kit: Protein C activator Protac® (DSM Pentapharm, Kaiseraugst, Switzerland), Lot number: 42335801; chromogenic substrate Pefachrome®PCa5297 (DSM Pentapharm), Lot number: 42133301; bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, Missouri, USA); and Tris, NaCl, HCl, NaOH, PEG-1500, and PEG-6000 (China National Medicines Corporation Ltd). (ii) Compared kit: Berichrom Protein C Activator (Siemens, Munich, Germany), Lot number: 546356; and Berichrom Protein C Substrate (Siemens), Lot number: 552922. (iii) other coagulation test kits: STA®-PTTA (activated partial thromboplastin time, Stago), Lot number: 257319; STA®-CaCl₂ 0.025 M (activated partial thromboplastin time, Stago), Lot number: 257116; STA®-Néoplastine® CI Plus (Prothrombin time, Stago), Lot number: 257377; STA®-Thrombin (Thrombin time, Stago), Lot number: 257402; STA®-Fibrinogen (Fibrinogen, Stago), Lot number: 257470; and D-dimer kit (Sun Biotech, Shanghai, China), Lot number: Z311, Z412. (iv) quality control and standard plasma: Protein C standard plasma (Siemens), Lot number: 503284A; normal-level control plasma (Siemens), Lot number: 507778C; and pathological-level control plasma (Siemens), Lot number: 507778C.

2.3 | Establishment of the new protein C detection system

2.3.1 | Reagent composition and concentration exploration

The protein C activator Protac® and the chromogenic substrate Pefachrome®PCa5297 were selected as the main components of R1 and R2, respectively. Optimal concentration ranges of the two were explored through concentration gradient experiments, and the concentration of buffer substances, and stabilizers in R1, R2, and sample diluent were also explored.

2.3.2 | Proportion of samples and reagents

According to the study on the concentration ranges of Protac® and Pefachrome®PCa5297 in this study, the range of the reaction volume ratio of the sample and reagents was preliminarily determined. The absorbance changes (ΔOD) of normal-level and pathological-level control plasma were examined under different ratios to compare the detection ability of different levels of plasma under different ratios. Standard plasma and a sample randomly selected from an apparently healthy person were simultaneously diluted four times (ratios: 1:1, 1:2, 1:4, and 1:8) to obtain four samples to be tested, respectively. XL-3200c was used to detect the linearity of different reaction volume ratios to evaluate whether it would affect the sensitivity and linearity of detection.

2.3.3 | Reaction time

The exploration of the reaction time was advanced based on the reaction proportion of samples and reagents obtained in the previous experiment. We added 30 μl sample into the reaction cup of XL-3200c, and then added 30 μl sample diluent to dilute it. Then, we put in 100 μl R1 to activate the PC in the sample and the mixture was incubated for 6, 8, 10, 12, and 14 min at 37°C, respectively. After the incubation was completed, 100 μl R2 was added for chromogenic reaction. The absorbance of the sample was detected as OD1 at 15 s of the reaction, and the absorbance was detected again as OD2 at 3, 5, 7, 9, and 11 min, respectively. The value obtained by subtracting the two was the ΔOD of the samples to be tested. The ΔOD of normal-level and pathological-level control plasma was examined under different incubation and detection time.

2.4 | Standard curves

2.4.1 | Newly built protein C detection system

To generate the new standard curve, standard plasma was diluted four times (S1–S4: 12.375%, 24.75%, 49.5%, and 99%). The four-parameter standard curve was generated by measuring and calculating the experimental samples' ΔOD by XL-3200c.

2.4.2 | Siemens protein C detection system

The standard curve was established by measuring and calculating ΔOD of the same standard plasma. Differently, the standard plasma was diluted six times (S1–S6: 5.2%, 12.4%, 24.8%, 49.5%, 99%, and 148.5%) and the six-parameter standard curve was obtained by the measurement of CN-6000.

2.5 | Performance evaluation

All performance evaluation tests mainly referred to the series of documents formulated by the Clinical and Laboratory Standards Institute (CLSI).

2.5.1 | Imprecision study

According to the CLSI EP5-A3 file,[7] normal-level control plasma, pathological-level control plasma, and normal mixed plasma were detected repeatedly for 20 consecutive times to obtain intra-day imprecision. For inter-day imprecision, the three levels of plasma were aliquoted and stored at −80°C until further analysis. We did one measurement each day, measured two batches a day, and repeated the measurement twice for each batch, for a total of 20 days of measurement. Imprecision was evaluated as the coefficient of variation (CV), which was calculated from the average ($\overline{x}$) and standard deviation (SD) of data.

2.5.2 | LoB and functional sensitivity

According to the CLSI EP17-A2 file,[8] the deionized water was selected as the blank sample. After continuous determination for 60 times, the value of the 95th percentile was calculated as LoB. We randomly selected a sample from samples that had been quantified by CN-6000 and identified as low PC activity (PC%: 33.51%); this was serially diluted to obtain six levels of specimens (1.09%, 2.09%, 3.39%, 6.82%, 15.97%, and 33.51%). Each level was measured twice a day for a total of 10 days, and we calculated the $\overline{x}$, SD, and CV of the data. The mean value of the low-level sample when the CV was closest to 20% was functional sensitivity.[9]

2.5.3 | Linear range test

According to the CLSI EP6-A file,[10] the linear range was assessed at all seven sites using sodium citrate plasma samples with one high-level sample (PC%: 144.40%) and one low-level sample (PC%: 22.1%). Each plasma sample was serially diluted from 7H+L to H+7L using the sample diluent, and we repeated the measurement twice for each diluted plasma to calculate the average of the data. We compared the mean level of instrumental results with the theoretical level, performed linear regression analysis, and made a scatter diagram (X: theoretical level, Y: instrumental level). Theoretical level = (CL × VL + CH × VH)/(VL + VH).

2.5.4 | Carryover rate (CR) test

Samples with high-level PC activity and low-level PC activity were randomly selected to perform carryover rate test. The high-level sample was repeatedly measured three times, and we then repeated the measurement of the low-level sample three times (recorded as H1 to H3 and L1 to L3). CR = |L1−L3|/(H3−L3) × 100%.

2.5.5 | Stability test

Three plasma samples with different activities were measured by the newly built PC detection system, and the test results were considered the initial measurement values. We put the PC detection kit at 37°C for thermal destruction. The sodium citrate plasma samples were tested for PC activity on day 1, day 3, day 7, day 10, and day 14 after thermal destruction. The relative deviation between the measured value after thermal destruction and the initial measured value was calculated, and the stability of the kit was considered to be favorable if the relative deviation was within 10%.

2.5.6 | Reference interval

A total of 152 anonymous serum samples from healthy subjects were used to establish the reference interval of this detection system. All the samples were collected and stored at –80°C for further testing. The normality test was performed on the test results. If the data were in line with normal distribution, $\overline{x}$ and SD of data were calculated to preliminarily establish the reference interval of this detection system. If not, it was necessary to sort the observed values of the reference individuals and we calculated the interquartile range after ranking them to establish the reference interval.[11,12]

2.6 | Method comparison

A total of 148 anonymous plasma samples from routine diagnostic testing including 80 apparently healthy subjects and 68 DVT patients were analyzed using the new protein C detection system. All the samples were stored at –80°C until tests were completed. Parallel testing of 148 samples was performed by using the Siemens detection system as the comparative procedure. We recorded the results of tests and evaluated the difference between the two detection systems by the paired sample t-test. The correlation equation and correlation coefficient were determined, and the consistency of the two detection systems was presented with the Bland-Altman plot. In addition, receiver operating characteristic (ROC) curves were used to analyze and compare the diagnostic efficiency of the two detection systems for DVT.[13,14]

2.7 | Clinical diagnostic value for DVT

A total of 148 anonymous plasma samples from 80 apparently healthy subjects and 68 DVT patients were subjected to routine coagulation tests, including activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT), fibrinogen (FIB), and D-dimer (DD) tests by using STA-RMAX. The test results of PC activity were obtained by the newly built detection system. ROC curve analysis was used to compare the sensitivity, specificity, negative likelihood ratio, and positive likelihood ratio of each test to evaluate their clinical diagnostic efficacy for DVT, as well as the diagnostic efficacy of each indicator carried out separately and in combination.

2.8 | Statistical analysis

The Kolmogorov-Smirnov test was used to test the normality of all measurement data. Continuous variables conforming to the normal distribution were expressed as $\overline{x} \pm$ SD, if not; they were expressed as median or interquartile range. For method comparison, an interrelationship of values obtained by the different PC detection systems was discerned by the Pearson correlation analysis. The difference between the two detection systems was calculated by the paired t-test. Bland-Altman plots were used to evaluate the consistency between the two detection systems. The clinical performance of different PC detection systems in distinguishing DVT patient samples from normal control samples was determined and compared through the ROC curve analysis. Statistical analysis and graphics were performed by SPSS version 26.0 (SPSS, Chicago, IL, USA), MedCalc version 17.0 (MedCalc Software bvba, Ostend, Belgium), and Origin 2018 (OriginLab, Hampton, VA, USA). p-values < 0.05 were considered statistically significant.

3 | RESULTS

3.1 | Establishment of the new protein C detection system

3.1.1 | Activator and substrate

After a series of concentration gradient tests, the optimal concentration ranges of R1 and R2 were determined; that is, the concentration range of the Protac® in R1 was 0.25–0.1 U/ml, and that of the chromogenic substrate Pefachrome®PCa5297 in R2 was 2.5–1 mM. The detection ability of PC activation degree was the best in the above concentration ranges. After serial dilution, the activity level of PC decreased gradually and the degree of linear fit was excellent.

3.1.2 | Buffer system

Tris-HCl was selected as the main buffer component of R1, R2, and sample diluent, and NaCl was also added to the above reagents and acted as an auxiliary buffer. The composition and concentration ranges of buffer affected the detection sensitivity and linearity. Optimal concentration ranges of Tris-HCl and NaCl were screened by observing the linearity of the ΔOD of samples detected by XL-3200c. The results showed that when 50–30 mM Tris-HCl and 0.9–0.09% mol/l NaCl were employed as buffer substances for reagents and sample diluent, the detection sensitivity for both normal-level and pathological-level control plasma was excellent. After serial dilution of the sample, the level of PC activity showed a fold-decreasing ratio and the linearity was very good.

3.1.3 | Stabilizer

The composition and concentration of stabilizer in R1, R2, and sample diluent should not only protect reagents from denaturation caused by interference factors but also ensure that the subsequent detection results are not affected. Three kinds of stabilizers, PEG-1500, PEG-6000, and BSA, were initially filtered for experiments.

Optimal composition and concentration ranges of stabilizers in R1, R2, and sample diluent were determined through a series of concentration gradient tests. We utilized 0.5% to 0.1% g/ml BSA as the stabilizer for R1 and sample diluent, and 1% to 0.1% g/ml PEG-6000 was selected as the stabilizer for R2. The level of PC activity showed a fold-decreasing ratio after serial dilution, and the degree of linear fit was excellent, indicating that the selected stabilizer components had little effect on the test results.

3.1.4 | The proportion of reagents and sample

The results showed that when S:R1:R2 = 60 μl (1:1):100 μl:100 μl, the degree of linear fit was the best. R^2 of ΔOD measured after dilution of the standard plasma could reach 0.9998 and that of the sample could reach 0.9996. It was also concluded through testing the quality control plasmas that this proportion had excellent detection sensitivity for both normal-level and low-level plasma samples, and the CV after repeated detection of both was less than 3%.

3.1.5 | Reaction time

The results showed that PC can be effectively activated to APC when the incubation time was 10 min (Figure 1A), and the chromogenic reaction reached the platform when the detection time was 5 min, after which the ΔOD no longer changed significantly (Figure 1B).

3.2 | Standard curves

3.2.1 | Newly built protein C detection system

The PC activity of standard plasma was 99%, and the calibration curve was fitted with four parameters, R^2 = 0.9996 (Figure 2A).

3.2.2 | Siemens protein C detection system

The PC activity of standard plasma was 99%, and the calibration curve was fitted with six parameters, R^2 = 0.9998 (Figure 2B).

3.3 | Performance evaluation

3.3.1 | Precision study

The total CVs of intra-day imprecision ranged from 1.01 to 2.10% (CV ≤ 3%) (Table S1). Also, for inter-day imprecision, the results showed satisfactory CVs ranging from 2.62 to 7.80% (CV≤10%) (Table 1).
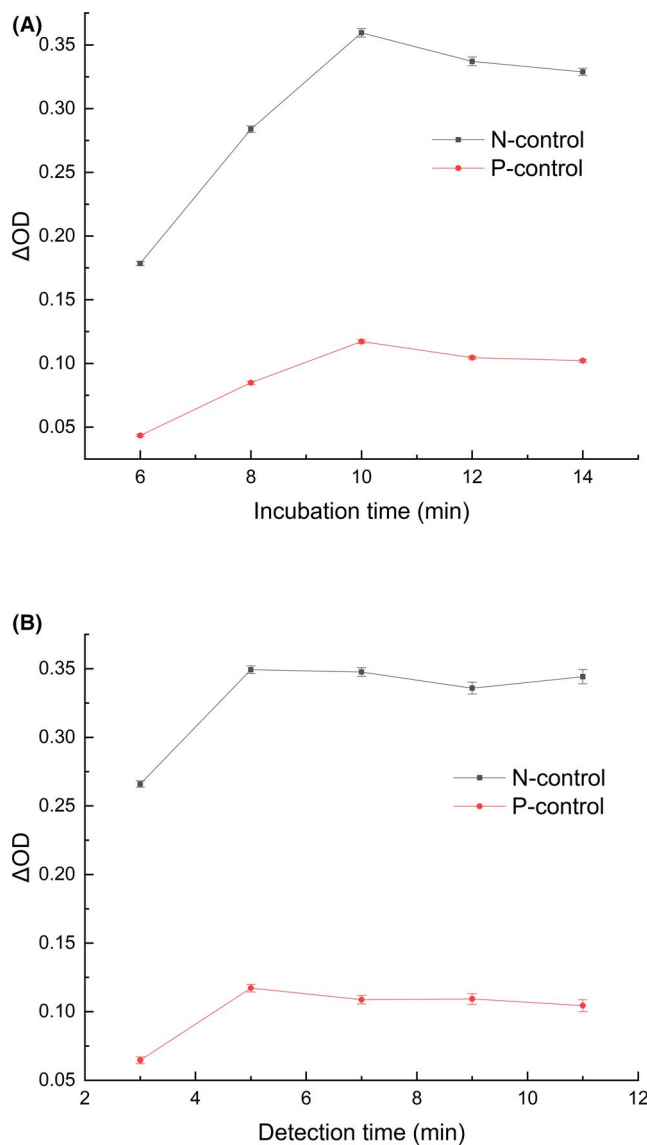

FIGURE 1 Reaction time. (A) Incubation time. (B) Detection time

3.3.2 | LoB and functional sensitivity

(i) LoB: The results of 60 repeated measurements of the blank sample were sorted from small to large, and the 95th percentile was 0.25%; hence, the LoB of this detection system for PC (%) was 0.25%.

(ii) Functional sensitivity: The PC activity of the selected sample was 30%. When the dilution ratio was 1:16, CV = 19.2%, which was the closest to 20%. The average value of PC(%) at CV = 19.61% was 2.09%, indicating that functional sensitivity of the detection system was 2.09% (Table 2).

3.3.3 | Linear range

There was no obvious outlier in the above test results. The regression equation obtained was as follows: $Y = 1.14151 + 1.00582X$,
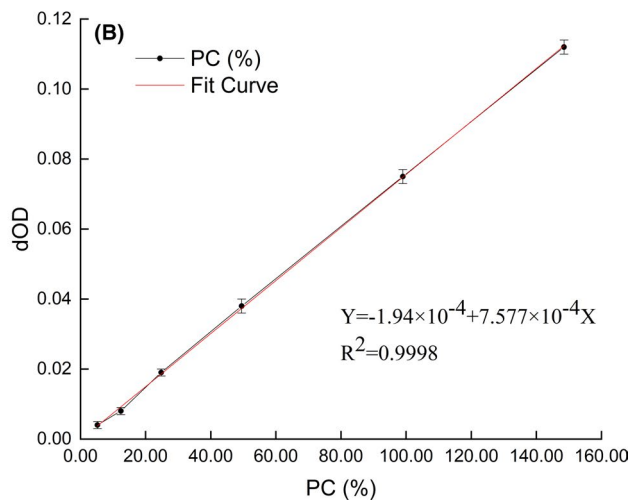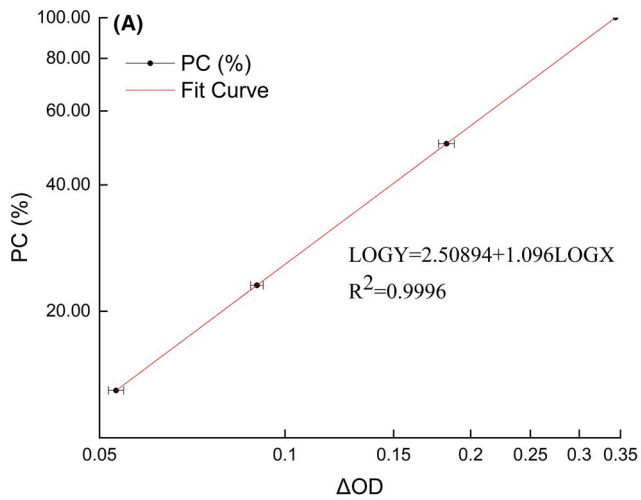
TABLE 1 Results of inter-day imprecision

	PC(%)		
	$\overline{x}$	SD	CV(%)
N control			
First test	110.72%	2.90%	2.62%
Second test	110.97%	3.11%	2.81%
P control			
First test	35.15%	2.46%	7.00%
Second test	34.16%	2.67%	7.80%
Mixed plasma			
First test	126.53%	3.92%	3.10%
Second test	127.51%	3.94%	3.09%

Note: CV, coefficient of variation; SD, standard deviation.
CV (%) = SD/$\overline{x}$ × 100%.

TABLE 2 Functional sensitivity

	PC(%)		
Dilution ratio	$\overline{x}$	SD	CV(%)
1:1	33.51%	1.14%	3.41%
1:2	15.97%	1.01%	6.30%
1:4	6.82%	0.72%	10.51%
1:8	3.39%	0.39%	11.60%
1:16	2.09%	0.41%	19.61%
1:32	1.09%	0.37%	34.00%

FIGURE 2 Standard curves. (A) Standard curve of the newly built protein C detection system (LOGY = 2.50894 + 1.096LOGX, R^2 = 0.9996). (B) Standard curve of Siemens protein C detection system ($Y = -1.94 \times 10^{-4} + 7.577 \times 10^{-4}X$, R^2 = 0.9998)
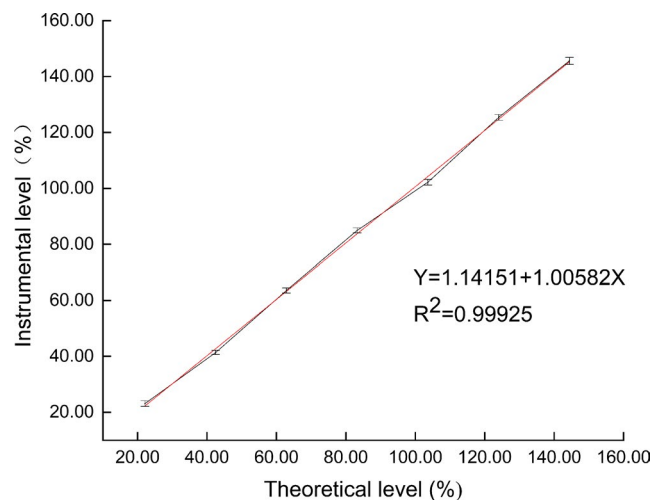

FIGURE 3 Linear range of the newly built protein C detection system. The black line represents the line of scattering; the red line represents the regression line

R^2 = 0.9993 ≥ 0.99, r = 0.986. The data were accepted as clinical linearity in the measuring range of 20 through 150% (Figure 3).

3.3.4 | Carryover rate

Carryover rate could be obtained by the following calculation: CR = |L1–L3|/(H3–L3) × 100% = |148.10%–20.33%|/(155.50%–20.33%) × 100% = 0.95 ≤ 3%.

3.3.5 | Stability test

For the kit after thermal destruction (37°C), the PC activity of samples showed an increase day by day. On Day 14, the relative deviation was 4.63%, 4.60%, and 4.72% for the PC activity that was low, medium and high, respectively, both of which were less than 5%, indicating satisfactory stability of the kit.

3.3.6 | Reference interval

The collected plasma samples of 152 apparently healthy persons were used to establish the reference interval, including 77 (51.97%) men and 75 (49.34%) women, aged 18 through 75 years. The results of PC (%) accorded with the normal distribution in the population, which showed that the reference range of PC (%) was 79.00 through 148.50% for this detection system.

3.4 | Method comparison

Sixty-eight DVT patients diagnosed for the first time were selected, including 35 (51.47%) men and 33 (48.53%) women aged 41 through 92 years. The control group consisted of 80 apparently healthy individuals, including 42 (52.5%) men and 38 (47.5%) women, aged 41 through 82 years. There was no statistically significant difference in the sex ratio and age composition between the two groups. The test results of the two test systems conformed to the normal distribution. The results of the paired sample t-test showed that there was no significant difference in the results of the two detection systems in both the control group and the DVT group ($p > 0.05$) (Table 3). A significant correlation was shown between the new detection system and Siemens detection system. The regression equation of the control group, which was obtained by the linear regression model, was $Y = 0.04 + 0.96021X$, $R^2 = 0.9771$, and the correlation coefficient between the two detection systems was $r = 0.989$ (Figure 4A). Also, the regression equation of the DVT group was $Y = 0.00221 + 1.00026X$, $R^2 = 0.9776$, and the correlation coefficient was $r = 0.986$ (Figure 4B). The Bland-Altman plots showed that the results of the two detection systems were highly consistent. In Figure 4C, three of the 80 points (3.75%) in the control group fall outside the 95% distribution range of the difference, and in Figure 4D, two of the 68 points (2.94%) in the DVT group fall outside the 95% distribution range of the difference. The proportions of outliers in the two groups were both less than 5%. ROC curve was adopted to compare the diagnostic capabilities of the two detection systems for DVT. The area under the curve (AUC) of the new PC detection system was 0.888, and the AUC of the Siemens detection system was 0.884. The results of the two were similar, thereby suggesting that both have excellent diagnostic proficiency for DVT (Figure 4E).

3.5 | Clinical diagnostic value for DVT

After the Kolmogorov-Smirnov test, the test results of PT, APTT, TT, FIB, and DD in the DVT group did not conform to the normal distribution, while the PC activity test results of the two groups were all in the normal distribution. After the Mann-Whitney U test, the differences in PT ($Z = 5.740$, $p = 0.000$), APTT ($Z = 4.118$, $p = 0.000$), TT ($Z = 3.414$, $p = 0.001$), FIB ($Z = 3.311$, $p = 0.001$), and DD ($Z = 5.498$, $p = 0.000$) between the two groups were statistically significant ($p \leq 0.01$) (Table S2). Moreover, the difference in PC activity ($t = -10.394$, $p = 0.000$) between the two groups was also statistically significant ($p \leq 0.01$) by the unpaired t-test (Figure 5). The AUC of APTT, PT, TT, FIB, DD, and PC was 0.697, 0.774, 0.663, 0.658, 0.763, and 0.888, respectively, which were statistically significant ($p \leq 0.01$) (Figure 6). Also, we also obtained the sensitivity, specificity, positive likelihood ratio, and negative likelihood ratio of each test at the maximum Youden index (Table 4). In addition, it was concluded by multivariate logistic regression analysis that the AUC of routine coagulation combined test (including APTT, PT, TT, FIB, and DD) was 0.913, and the AUC of routine coagulation test combined with PC (%) detection was 0.966 (Table S3). Compared with single coagulation index detection, the AUC of the combined detection of coagulation indicators was significantly improved.

4 | DISCUSSION

In recent years, there has been a deeper understanding of diseases related to the PC system with improvement in diagnostics. However, current research and development of protein C detection reagents in China are still at a relatively nascent stage. This may be due to the lack of clinical development of tests for detection of PC activity in China, which leads to insufficient understanding of the test. In 2017, a survey of 1500 laboratories by the National Center for Clinical Laboratories of China showed that less than 3% of laboratories conducted plasma PC activity testing. In order

TABLE 3 Difference analysis between the two detection systems

| Group | Detection system | $\bar{x} \pm SD\,(\%)$ | $d(\%)$ | | t | p |
			$\bar{x} \pm SD$	95% CI		
Control group (n = 80)	Newly built	114.56 ± 17.29	0.49 ± 2.68	−0.11–1.08	1.627	0.108
	Siemens	115.05 ± 17.89				
DVT group (n = 68)	Newly built	84.19 ± 18.08	0.29 ± 3.02	−0.44–1.02	0.800	0.426
	Siemens	84.48 ± 18.23				

Note: The difference analysis of the test results of the two detection systems was completed by the paired sample t-test. p-values < 0.05 were considered statistically significant.
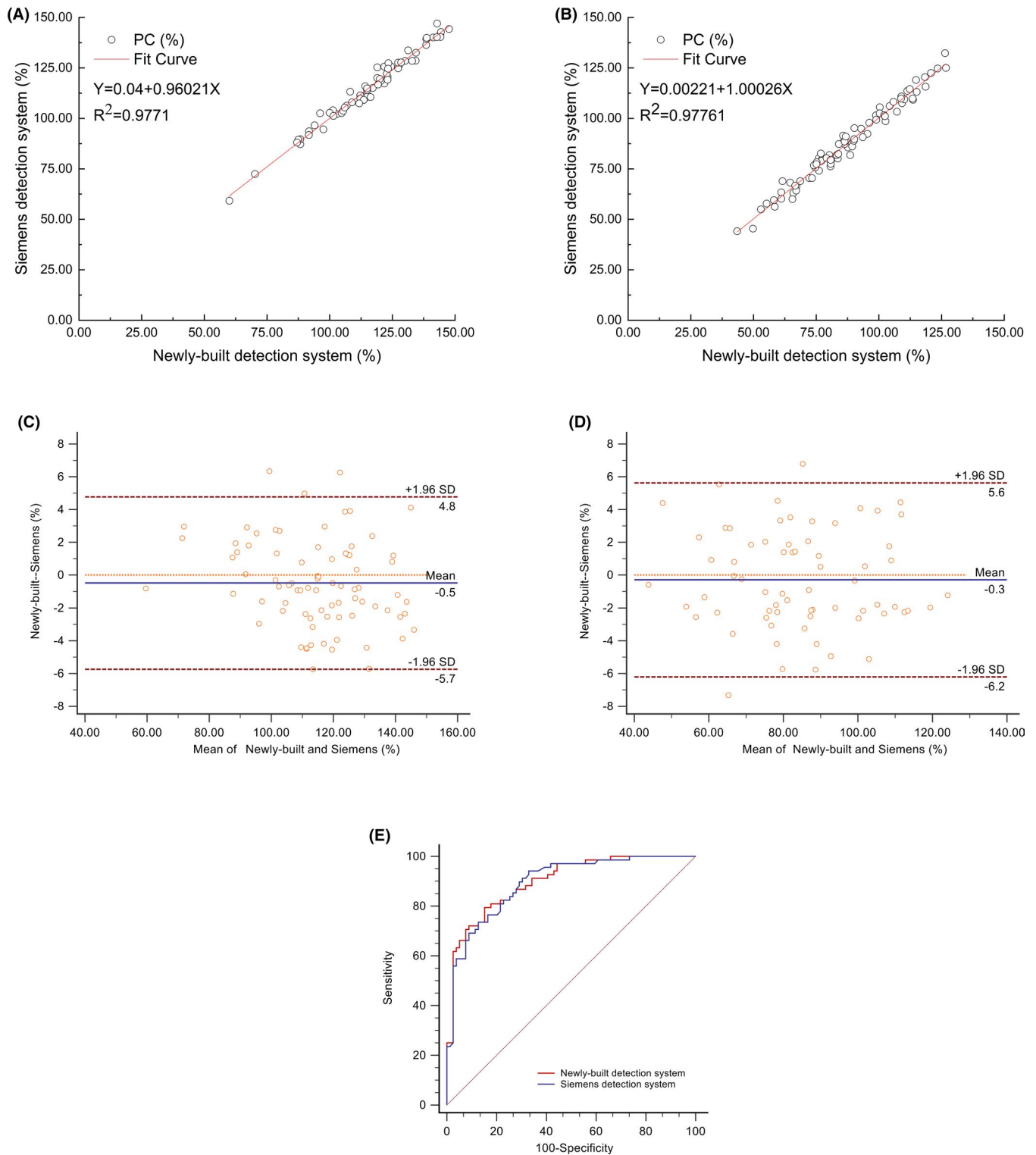
FIGURE 4 Method comparison. (A) Correlation analysis of the two detection systems in the control group (n = 80). (B) Correlation analysis of the two detection systems in the DVT group (n = 68). (C) The Bland-Altman plots in the control group (n = 80). (D) The Bland-Altman plots in the DVT group (n = 68). (E) ROC curves of the two detection systems

to ameliorate this situation, this study established a PC detection system based on the chromogenic substrate method, conducted preliminary performance evaluation, and comparison with Siemens detection system.[15,16]

The chromogenic substrate method has been recommended as the preferred screening experiment for PC deficiency due to its advantages of excellent sensitivity, stability, reproducibility, and strong anti-interference ability.[6] After investigation and exploration, we chose
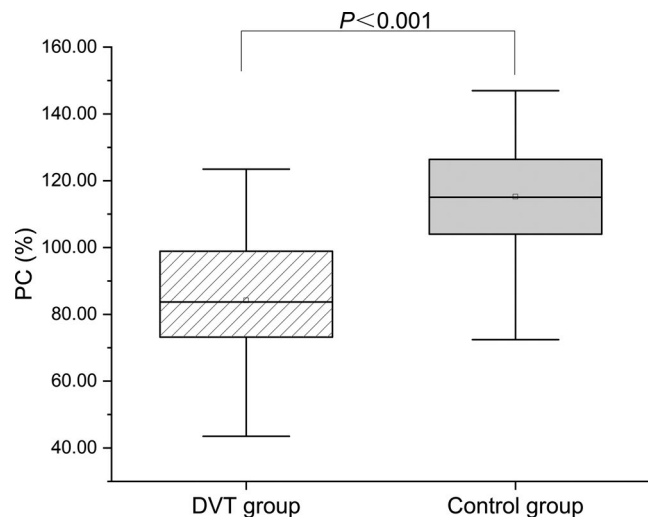
FIGURE 5 PC activity in DVT and control groups. Plasma PC levels in DVT patients (84.19 ± 18.08%) were significantly decreased when compared to the healthy subjects (114.56 ± 17.29%, *p* = 0.000; unpaired *t*-test)

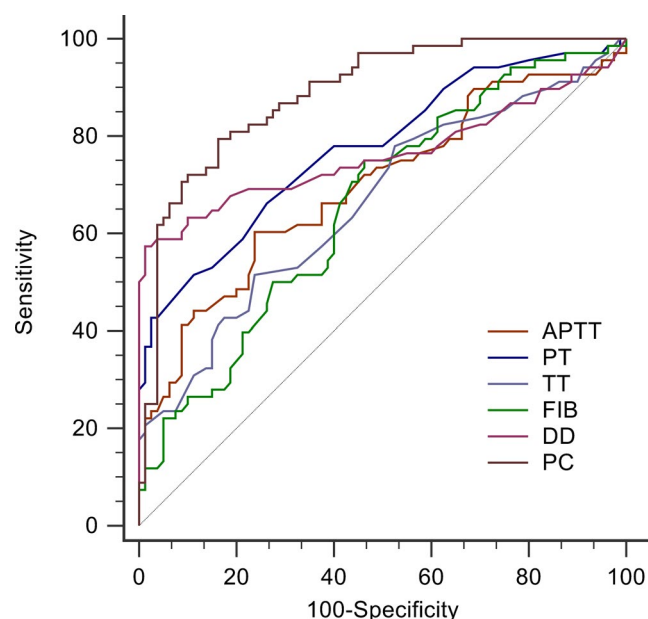**FIGURE 6** ROC curves of APTT, PT, TT, FIB, DD, and PC

Protac® as the activator of PC and Pefachrome®PCa5297 as the chromogenic substrate. Optimal parameters for them were obtained through a series of concentration gradient experiments. Protac® is a single-chain protease that can quickly activate PC; it was first isolated from the venom of the American Copperhead Agkistrodon by Stocker et al. in 1986.[17] Compared with the physiological activator of PC, Protac® has higher activation specificity and sensitivity and does not require the participation of cofactors.[18] The composition of Pefachrome®PCa5297 is Pad-Pro-Arg-pNA•AcOH; it has a molecular weight of 576.6 g/mol and can have a stable and efficient chromogenic reaction with APC. In addition, we added stabilizers to the reagents in order to reduce the influence of interfering enzymes and other factors in the plasma on the stability of the reagents. We finally chose BSA as the stabilizer for R1 and sample diluent, and PEG-6000 as the stabilizer for R2. BSA is an albumin in bovine serum, which can reduce enzyme denaturation and some unfavorable environmental factors, such as heating, surface tension, and chemical factors. PEG-6000 is non-toxic and non-irritating, and is widely adopted in various pharmaceutical preparations. It can be used as a stabilizer to regulate the melting point and viscosity, as well as an excipient, and aids in the preservation of reagents. Also, the study further optimized reaction time and the proportion of samples and reagents to make it amenable to XL-3200C, thereby establishing a plasma PC activity detection system and evaluating the performance after referring to the CLSI documents. This detection system showed high precision, sensitivity, specificity, and a wide linear range.

PC deficiency is a genetic risk factor for DVT that cannot be ignored. Studies have shown that the incidence of DVT in the West is 1/1000; of this, 12–15% of thrombotic diseases are related to the abnormal level of antithrombin III, PC, PS, and plasminogen.[19] The age of onset of VTE caused by heterozygous PC deficiency is earlier than that of onset of general thrombosis, and the clinical symptoms are more severe with proclivity to recurrence.[5,20] PC deficiency is a relatively common genetic disease in China with an associated proneness to thrombosis. A study that tested PC activity in 202 patients with venous thromboembolism (VTE) showed a significant decrease in PC activity in 34 (17%) patients.[21] In order to explore the clinical application value of the newly built PC detection system, we tried to apply it to the detection of plasma PC activity in DVT patients. In this study, the results of routine coagulation tests (including APTT, PT, TT, FIB, and DD) and PC activity were all statistically significant

TABLE 4 Diagnostic efficacy indicators at the maximum Youden index

Test	Cut-off value	Youden index	Sensitivity (%)	Specificity (%)	LR+	LR−
APTT(s)	≥36.5	0.3654	60.29	76.25	2.54	0.52
PT(s)	≥13.1	0.4022	51.47	88.75	4.58	0.55
TT(s)	≥17.5	0.2772	51.47	76.25	2.17	0.64
FIB(g/L)	≥2.95	0.2875	75.00	53.75	1.62	0.47
DD(g/L)	≥660	0.5610	57.35	98.75	45.88	0.43
PC(%)	≤100.38	0.6316	79.41	83.75	4.89	0.25

Note: LR+: positive likelihood ratio; LR−: negative likelihood ratio.

between the DVT group and apparently healthy people ($p \leq 0.01$). It is worth noting that the diagnostic sensitivity of PC for DVT in this study was 79.41%, which was higher than all other coagulation indicators. In addition, we also applied logistic regression analysis to evaluate the efficacy of combined detection of multiple indicators, which showed that the AUC of combined detection of multiple coagulation indicators was significantly higher than that of a single indicator. The above results further confirmed that the decrease in PC activity was a non-negligible risk factor for DVT, and the combination of PC activity detection and routine coagulation tests could effectively improve the diagnostic sensitivity and specificity for DVT. For people with recurrent thromboembolic diseases and familial genetic predispositions, screening PC content and activity, and even gene mutation analysis can help clinicians find the cause of DVT in a timely manner, guide timely medication, and reduce the incidence and fatality rate due to DVT.[22,23]

The PC detection system established in this study has the following advantages: (i) Compared with the detection kit based on the coagulation method, the chromogenic substrate method is not easily affected by some interfering enzymes and anticoagulant drugs *in vivo*, and has higher stability and precision.[13,15] (ii) The reaction time is moderate, and the entire detection process only takes 15 min. (iii) The reagent production cost is lower, which can effectively reduce the burden on patients and facilitate the development of PC activity testing in the clinic. (iv) The measurement range is wide, and the correlation coefficient in the linear range is 0.986, which is better than Siemens ($r = 0.975$). (v) The precision is higher. The intra-day imprecision CV of the kit is ≤3%, and the inter-day imprecision CV is ≤8%, these are better than the Siemens detection system (the intra-day imprecision CV is ≤5%, and the inter-day imprecision CV is ≤10%).[24] (vi) The diagnostic efficiency for DVT is high. ROC curves show that the AUC of the detection system established in this research is 0.888, which is slightly higher than the Siemens detection system. In addition, in order to verify the reliability of this testing system, we conducted correlation and consistency evaluation between the newly built PC testing system and Siemens testing system. The results showed that whether it is used to detect PC activity of apparently healthy people or DVT patients, the results of this detection system correlated highly with and were consistent with the comparison system.

However, this study also has some limitations. For example, the lyophilized conditions of the reagents have not been explored and the established reference interval was relatively crude. The next step would therefore be to explore optimal lyophilized parameters and increase the sample size to establish a more detailed reference interval according to age and sex.

5 | CONCLUSION

The newly built protein C detection system has high sensitivity, strong stability, and wide linear range. It is worth mentioning that its test results have a good correlation with the current commercial PC detection system, which also confirms the reliability of the PC detection system in this study. In addition, it has been applied to the clinical diagnosis of DVT patients, and satisfactory results have been obtained.

CONFLICT OF INTERESTS
Anshan Ge and Hui Rong are employees of Beijing ZONCI Technology Development Co., Ltd and Anshan Ge hold stocks/shares in Beijing ZONCI Technology Development Co., Ltd. The sponsor was involved in study design but played no role in the collection and interpretation of the data and writing of the article.

AUTHOR CONTRIBUTIONS
Limin Lun, Wenfei Lu, and Anshan Ge conceived and designed research. Wenfei Lu, Hui Rong, and Chao Zhu performed the research. Wenfei Lu and Jinxia Zhao analyzed the data. Wenfei Lu wrote the article. Limin Lun and Jinxia Zhao revised the article. All authors approved the final version of the article.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE
Research involving human subjects complied with all relevant national regulations and institutional policies, is in accordance with the tenets of the Helsinki Declaration (as revised in 2013), and has been approved by the Ethics Committee of the Affiliated Hospital of Qingdao University (number QYFYWZLL26452).

DATA AVAILABILITY STATEMENT
All relevant data are within the article, and no additional data are available.

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SUPPORTING INFORMATION

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