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# Development of a chemiluminescent immunoassay based on magnetic solid phase for quantification of homocysteine in human serum

Yankui Liu[1,2†], Yaru Li[1†], Rong Wang[1†], Shuning Chen[1], Ning Sun[2*] and Xiaowei Qi[1*]

## Abstract

**Background** Homocysteine (HCY) is a sulfur-containing amino acid that is an independent or important risk factor for the occurrence of many chronic diseases and is one of the most important indicators for determining health risks. However, existing HCY detection methods do not meet the requirements of clinical diagnosis. Therefore, there is an urgent need to establish new detection methods to meet the needs of clinical detection.

**Results** In this study, we used the principle of competitive method to establish a new method for the determination of HCY in human serum using a chemiluminescent enzyme immunoassay in conjunction with a chemiluminescent assay instrument that uses magnetic microparticles as the solid phase of the immunoreaction. The established method achieved satisfactory results in terms of minimum detection limit, specificity, accuracy, and clinical application. The limit of detection was 0.03 ng/mL. The intra-assay coefficient of variation (CV) was 1.94–5.05%, the inter-assay CV was 2.29–6.88%, and the recovery rate was 88.60–93.27%. Cross-reactivity with L-cysteine ranged from 0.0100 to 0.0200 µmol/L, and cross-reactivity with glutathione ranged from 0.0100 to 0.200 µmol/L, all of which were less than the limit of detection (LoD) of this method. The linear factor R of this method was greater than 0.99.

**Conclusions** In summary, the developed method showed a good correlation with the product from Abbott. A total of 996 clinical patients with cardiovascular diseases were evaluated using the method developed in this study.

**Keywords** HCY, Cardiovascular diseases, Chemiluminescent immunoassay

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## Background

The human body cannot directly ingest or synthesize homocysteine (HCY) [1]. HCY is an intermediate product produced during the metabolism of methionine and cysteine [2]. Methionine, an essential amino acid derived from dietary proteins such as poultry, meat, eggs, seafood, and dairy products, is an exclusive dietary source of HCY [3]. The liver plays a pivotal role in regulating HCY levels, as it produces enzymes essential for methionine metabolism [4, 5]. Methionine is initially transformed by methionine adenosyltransferase into S-adenosylmethionine (SAM) [6], a highly energetic compound, with adenosine triphosphate supplying the adenosyl group. Subsequently, SAM donates a methyl group via methyltransferase to form S-adenosylhomocysteine (SAH) [6]. Finally, SAH hydrolase catalyzes the conversion of SAH to HCY [6]. Therefore, the equilibrium of products and metabolic balance of HCY is crucial for maintaining human homeostasis.

Excessive or insufficient HCY concentrations have been consistently shown to negatively affect human health [3, 7]. Recent research findings have indicated a statistically significant correlation between elevated HCY levels and a range of health issues [7], including disorders in the cardiovascular [8], gastrointestinal [9], urinary [10], skeletal [11], and autoimmune systems [12], as well as cancer [13]. Several methods have been developed to detect serum HCY levels in clinical settings [14], including gas chromatography-mass spectrometry [15], liquid chromatography-mass spectrometry [16], radioenzyme analysis [17], capillary electrophoresis [18], high-performance liquid chromatography (HPLC)- fluorescence detection [19], enzyme-linked immunosorbent assays [20], chemiluminescent microparticle immunoassays [21], fluorescence polarization immunoassays [22], and HPLC [23]. Nevertheless, no universally accepted method of detection exists because these methods are usually limited by expensive instruments and detection reagents or insufficient sensitivity [3]. Therefore, a method that can rapidly and accurately detect HCY based on simple operation is urgently needed.

Since magnetic particle-based chemiluminescence immunoassay is based on a liquid-phase reaction that allows for sufficient contact between the substrate and reactants, it has clear advantages in terms of linear range, sensitivity, precision, accuracy, and good detection stability [24–29]. Furthermore, detection is rapid and convenient with fully automated instruments. Given these advantages, a sandwich chemiluminescence assay was established to detect HCY levels in human serum.

## Methods

### Reagents and materials

The anti-SAH-AP conjugate is a conjugation reaction between the SAH monoclonal antibody and alkaline phosphatase (AP), purified to form a conjugation, dithiothreitol (DTT), and recombinant S-adenosyl-L homocysteine hydrolase (SAHase), obtained from ZECEN Biotech Co., Ltd (Taizhou, Jiangsu Province, China). Magnetic particle conjugates formed by linking SAH antibodies to magnetic particles were supplied by ZECEN Biotech Co., Ltd. (Taizhou, Jiangsu Province, China). The HCY recombinant antigen was supplied by ZECEN Biotech Co., Ltd. (Taizhou, Jiangsu Province, China). BSA was purchased from ROCHE (Basel, Switzerland). Carboxyl magnetic beads were purchased from BIOMAG (Wuxi, Jiangsu Province, China). The manufacturer and model of the chemiluminescence analyzer utilized was the CIA1200 Full-auto Chemiluminescence Analyzer (ZECEN Biotech Co., Ltd, Taizhou, Jiangsu Province, China).

### Reagent component preparation

#### *Characterization of anti-SAH-AP conjugate, SAHsae, and reducing agent*

Anti-SAH-AP (2 μg/mL): add SAH monoclonal antibody conjugate (SAH antibody alkaline phosphatase AP) to buffer containing BSA (pH = 8.0) to prepare SAH antibody reagent. Reducing agent (2 μg/mL): prepared by adding dithiothreitol (DTT) to the citrate buffer solution. SAHase (2 μg/mL): recombinant S-adenosyl-L-hydrocysteine hydrolase (SAHase) was prepared by adding it to tris containing buffer. Magnetic particle reagent (3 mg/mL): magnetic particles and SAH connectors in BSA buffer (pH = 8.0). The concentration and recovery rate of the antibodies were calculated by measuring their absorbance values at 280 nm using a UV spectrophotometer. The selected concentration of anti-SAH-AP was 2.0 mg/mL, the reducing agent (DTT) concentration was 3. 0 mg/mL, and the SAHase concentration was 3.0 mg/mL. The recovery rates were 100.70%, 102.90%, and 105.13%, respectively.

### Reaction condition optimization

This reagent kit adopts the principle of competition method, using magnetic microparticles as the solid phase of the immune reaction, in addition to using chemiluminescence enzyme-linked immunosorbent assay method in combination with chemiluminescence measuring instruments to determine the HCY concentration in human serum.

The technical principle is that bound or dimerized HCY (oxidized) is reduced to free HCY by the action of dithiothreitol (DTT). In the presence of sufficient

adenosine, free HCY is converted to S-adenosine homocysteine (SAH) by recombinant S-adenosine homocysteine hydrolase (rSAHHase). SAH competes with magnetic particle-labeled SAH to bind to alkaline phosphatase (AP)-labeled anti-SAH monoclonal antibodies. It directly precipitates in an external magnetic field to separate the complexes formed by immune reactions from other unbound substances. The precipitated complex was cleaned by adding an enzyme-catalyzed chemiluminescent substrate, which is catalytic cracked under the action of the enzyme, forming an unstable excited state intermediate. When the excited state intermediate returns to the ground state, it emits photons, forming a luminescent reaction. The luminescence intensity of the reaction is measured using a luminescent instrument. Within the measurement range, luminescence intensity is inversely proportional to the concentration of HCY in the sample. The improved Four-Parameter Logistic (4PL) Model can quantitatively calculate the HCY concentration in the test sample. In this study, factors such as magnetic particle, anti-SAH-AP, and DTT concentrations and incubation time were optimized.

For the details of the 4PL model, it is defined by the following equation:

$$y = \frac{A_1 - A_0}{1 + \left(\frac{x}{x_0}\right)^p} + A_0$$

Curve Shape: S-shaped, either increasing or decreasing.

A1: The maximum value of y as x approaches infinity or negative infinity.

A0: The minimum value of y as x approaches infinity or negative infinity.

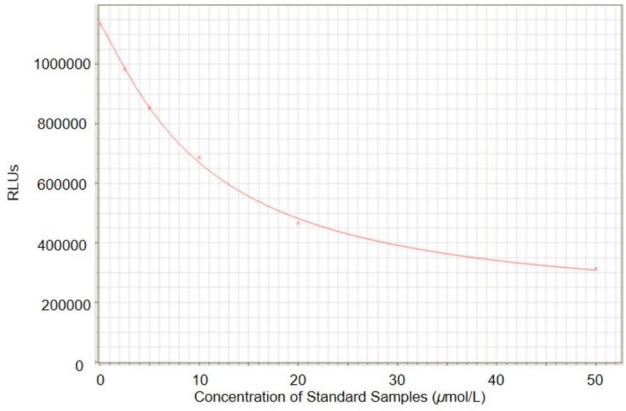
X: The inflection point of the curve.

P: Related to the slope of the curve at the inflection point.

Based on the above formula, we will substitute the following data for calculation.

| Concentration of Standard Sample | RLUs |
|---|---|
| 0 μmol/L | 1,137,651 |
| 2.5 μmol/L | 983,113 |
| 5 μmol/L | 852,651 |
| 10 μmol/L | 686,961 |
| 20 μmol/L | 466,081 |
| 50 μmol/L | 313,807 |

We obtain the following curve:




And obtain the following formula for the concentration calculation of subsequent test samples:

$$\mathrm{Y} = 1135118.3913 + (-953720.5979) / (1 + \mathrm{EXP}(-(-2.8019 + 1.1945 * \mathrm{Ln}(\mathrm{X}))))$$

**Method procedure**

First, add 30 μL of calibration, test samples, or quality control products into the reaction, then add 15 μL DTT, mix well, and react at 37 ℃ for 5 min. Then, add 15 μL SAHase into the reaction and mix well; add 30 μL magnetic particle reagent and 30 μL anti-SAH-AP, mix, and react at 37 ℃ for 30 min. Then, add 300 μL of cleaning solution for the chemiluminescence system and mix well. Repeat this step three times for magnetic separation and cleaning of the reactants. Finally, add 200 μL chemiluminescent substrate solution for automatic immunoassay for luminescence value detection, and concentration values are calculated if the calibrator curve is available.

**Sample collection**

The selection criteria are as follows: A specific proportion of samples exhibiting aberrant values outside the established reference range and a corresponding proportion of samples displaying normal values are to be identified, with a minimum total sample size of 120 and a minimum of 30% of the total number of samples exhibiting aberrant values (a minimum of 36 samples); this is to be achieved by utilizing the control product. Samples with abnormal values are expected to display a range of HCY levels. All selected samples must meet the requisite information for this validation process. It is imperative that specimens exhibiting severe hyperlipidemia, hemolysis, or jaundice be excluded from the study. Specimens that do not align with the clinical situation, as indicated by the comparative reagent assay results, are excluded from further analysis.

To establish the reference interval for this kit, 800 serum specimens from healthy (or normal) individuals

were collected from a health-testing center within our hospital. Of these, 400 were from individuals aged over 70 years, and 400 were from individuals aged under 70 years. The 95th percentile of the test values for the normal human samples was calculated as the reference value, with a lower limit of 2.5% and an upper limit of 97.5%.

## Performance test method

### Limit of detection (LoD)

Using zero concentration calibration samples or sample diluents for detection, repeat the measurement 20 times to obtain the relative luminescence value (RLU) of the 20 measurement results. Calculate the average value (M) and standard deviation (SD) to obtain M-2SD. Based on the concentration RLU value results between point A calibration samples and adjacent point B calibration samples, perform a two-point regression fitting to obtain a first-order equation. Substitute the RLU value of M-2SD into the above equation and calculate the corresponding concentration value, which is the lowest detection limit.

### Precision and recovery

Take quality control samples 1 and 2 from the same batch of reagent kits as the test samples. Each test is repeated ten times, with a coefficient of variation (CV) not exceeding 8% for the intra-assay. Using three different batches of reagent kits and following the instructions, use a chemiluminescence analyzer to measure the samples separately 30 times (10 times per batch of reagent kits). The CV between the three batches of reagent kits should not exceed 15%. Add 10 μL 50 μmol/L sample A to 90 μL 2.5 μmol/L sample B, and the volume of A added should not exceed 10% of the total volume (A+B), and the recovery rate was calculated.

### Accelerated stability

After determining all the components in the reagent kit, prepare small samples and divide them into five portions (100 tests). Place them in a 37 ℃ incubator for 1, 2, 3, and 7 days, and measure the performance of the reagent kit at 37 ℃ for 0 h, 1, 2, 3, and 7 days, respectively. Conduct performance tracking experiments on three batches of reagent kits under long-term actual storage conditions (2 ℃ to 8 ℃). Determine the shelf life of the three batches of reagent kits based on their stability under storage conditions. The appearance and performance indicators (linearity, accuracy, minimum detection limit, and repeatability) of the test kit are tracked and tested by placing it under conditions of 2 ℃ to 8 ℃ for 10 months and then inspecting it once every 3, 6, 9, and 10 months.

### Linearity

Dilute high-value samples close to the upper limit of the linear range to at least five concentrations in a certain proportion, with low-value samples approaching the lower limit of the linear range. According to the instructions of the reagent kit, repeat the test three times for each concentration of the sample, calculate the average value, fit the average value and dilution ratio using the least squares method, and calculate the linear correlation coefficient *r*. The linear range of the HCY reagent kit is 0.4–50.0 μmol/L. Within this linear range, the correlation coefficient *r* between the reference and test curve should be less than 0.9900.

### Method comparison

The established method was compared with an on-market CLIA method from Abbott. The sample size used to compare with Abbott was 240 and ranged from 4.04 μmol/L to 40.98 μmol/L.

## Results

### Serum HCY chemiluminescence assay development

This method adopts the principle of competitive method, using magnetic microparticles as the solid phase of the immunoreaction and the chemiluminescent enzyme immunoassay method with a chemiluminescent measuring instrument to determine the level of free HCY in human serum. A stable and reliable experimental procedure was determined by optimizing the SAH antibody coupler, reductant DTT, and SAH hydrolase involved in the method and systematically optimizing the sample volume, reaction time, and temperature (Fig. 1).

### Method performance

The 4-parameter logic function method was used to fit the standard curve of the signal with HCY concentration. The following series of concentrations of HCY were used as a standard curve (A-F): 0, 2.5, 5.0, 10.0, 20.0, and 50.0 μmol/L. The LoD is 0.3 μmol/L. Notably, the coefficient of variation (CV) is a statistic employed to ascertain the extent of dispersion of the constituent data points a dataset around its mean; this is a valuable metric in the context of comparative analysis as it allows for the assessment of variation across data series, even when there is considerable divergence in their respective means. Therefore, the precision was tested; the intra-assay CV % was 1.94 – 5.05%, and the Inter-assay CV % was 2.29 – 6.88%; the results are shown in Table 1. Considering that the kit is required to demonstrate an intra-assay CV of less than 8% and an inter-assay CV of less than 15%, the data indicate that the developed HCY kit has satisfactory accuracy and reproducibility, highlighting its commendable analytical performance and capacity to meet the demands of clinical practice.

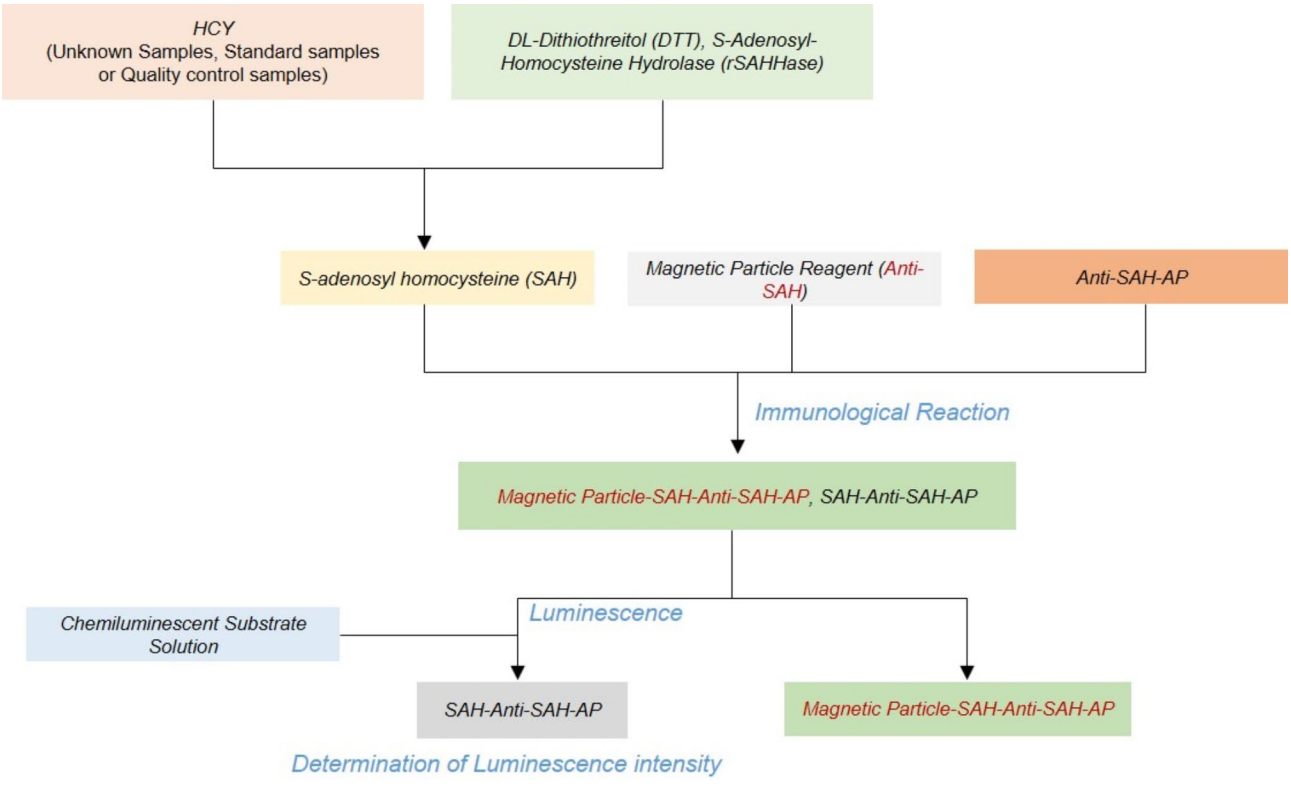



**Fig. 1** Scheme of the applied procedure

**Table 1** Precision test results

| Samples | Intra-assay CV (n = 20) | | | Inter-assay CV (n = 20) | | |
|---|---|---|---|---|---|---|
| | Mean (μmol/L) | SD | CV (%) | Mean (μmol/L) | SD | CV (%) |
| 1 | 275.24 | 13.91 | 5.05% | 269.38 | 18.53 | 6.88% |
| 2 | 875.11 | 16.94 | 1.94% | 881.06 | 20.22 | 2.29% |

**Table 2** Optimization of the antibody concentration

| Anti-SAH-AP (μg/mL) | 0.1 | | | 2.0 | | | 5.0 | | |
|---|---|---|---|---|---|---|---|---|---|
| DTT (μg/mL) | 0.1 | 2.0 | 5.0 | 0.1 | 2.0 | 5.0 | 0.1 | 2.0 | 5.0 |
| Standard 1 (0 μmol/L) | 349377 | 360657 | 355173 | 360610 | 727219 | 884461 | 356137 | 727837 | 958345 |
| Standard 2 (2.5 μmol/L) | 279598 | 290191 | 279092 | 298932 | 554226 | 625298 | 308952 | 543313 | 830502 |
| Standard 3 (50 μmol/L) | 53040 | 64025 | 66878 | 69015 | 95542 | 199266 | 71169 | 103976 | 329685 |
| SAHase (μg/mL) | 0.1 | | | 2.0 | | | 3.0 | | |

## Advice on developed method

### *Optimization of the sample volume and antibody concentration in the reagent*

As shown in Table 2, a series of concentrations of anti-SAH-AP and DTT were tested. Additional free antibodies were added to the reaction system to consume excess antigens in the samples. As shown in Fig. 2, the greatest discrepancy in absorbance values was observed between the three standards when a sample volume of 30 μl was employed. Therefore, the sample volume was optimized at 30 μl. Additionally, the DTT, SAHase, and anti-SAH-AP reaction dosages were optimized (These components were explored by taking 15 μl at indicated concentrations in the table). As listed in Table 2, at a dose of 2.0 μg/mL, the difference in absorbance values between the three standards was the most significant, and the linearity was optimal (The units of numbers in the table are μg/mL). Finally, 30 μL anti-SAH-AP (2.0 $\mu$g/mL), 15 μL DTT (2.0 $\mu$g/mL), and 15 μL SAHase (2.0 $\mu$g/mL) were selected as the optimal formation, as shown in Table 3.

### *Optimization of the incubation time*

The anti-SAH-AP and sample incubation times were assessed. In addition to the incubation time, the procedure was performed as described in the Methods. The RLUs increased with increasing incubation time and

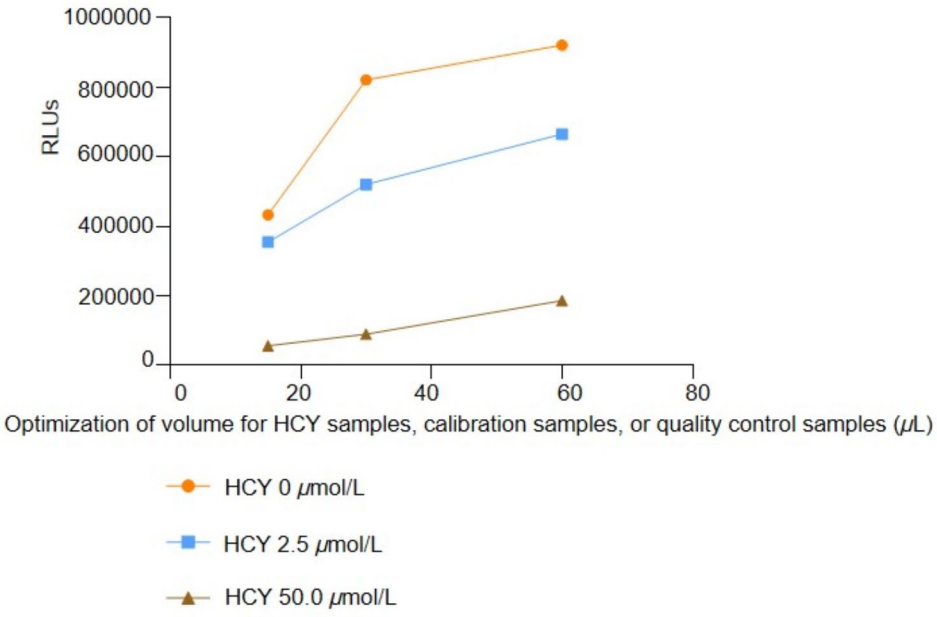



**Fig. 2** Optimization of sample volume

**Table 3** Optimization of the HCY anti-reagent volume

| Anti-reagent volume | 15 μL + 15 μL + 30 μL | 30 μL + 30 μL + 60 μL | 60 μL + 60 μL + 120 μL |
|---|---|---|---|
| Standard 1 (0 μmol/L) | 818025 | 838869 | 849388 |
| Standard 2 (2.5 μmol/L) | 517944 | 531142 | 537802 |
| Standard 2 (50 μmol/L) | 88956 | 91222 | 92366 |

remained unchanged after 30 min when the reaction reached dynamic equilibrium. Owing to time-saving clinical requirements, we selected a 30 min incubation time. The signal results are shown in Fig. 3.

### Reaction flow and reaction parameters

To this end, the aforementioned data illustrates that the following reaction flow applies to this kit: the user is required to select the corresponding test item on the instrument's operation interface, generate the work list, select 'start testing,' and then allow the instrument to perform automatic testing in accordance with the item's set reaction flow and reaction parameters. The details are presented in Table 4.

### Comparison of methods

In this study, 240 serum samples were analyzed using both the developed method and the Abbott method, which is a clinically utilized detection method for HCY in the context of cardiovascular disease diagnosis [21]. The test results were regressed using the least-squares regression equation, and the correlation coefficient was computed. Data are shown in Fig. 4. The test results show good agreement between the developed and compared methods, with a correlation coefficient ($R^2$) of 0.95, and the difference in the test and mean values indicated a slight bias between these methods. Notably, the discrepancies in HCY values observed between the two methods were predominantly evident in samples from patients with elevated HCY levels. Based on our experience in the development of the kits, we postulate that the potential causes for this may include epitopes recognized by the antibodies, as well as elevated levels of other substances that may interfere with the detection of HCY.

### Clinical study

The most crucial element in evaluating the efficacy of the kits we create is their precision in a clinical context. First, to determine the reference interval of the kit, 800 serum samples were collected from healthy individuals at our hospital's Health and Wellness Center. Of these, 400 were from individuals aged over 70 years, and 400 were from individuals under 70 years old. Furthermore, a bilateral 95% reference value was calculated using the assay values of normal human samples, with a lower limit of 2.5% and an upper limit of 97.5%. The final reference values for this kit were determined as ≤70 years: 4–14 μmol/L; >70 years: 6–20 μmol/L. Given that an elevated HCY level has been identified as a key biomarker for cardiovascular disease [8, 30], we collected serum samples from 996 patients with cardiovascular disease to evaluate the precision of our kit. As shown in Table 5, the sensitivity of our kit for the detection of disease was 93.4% (930 996), suggesting that our kits have the potential for clinical use. Consistent with a previous study [31], we also observed

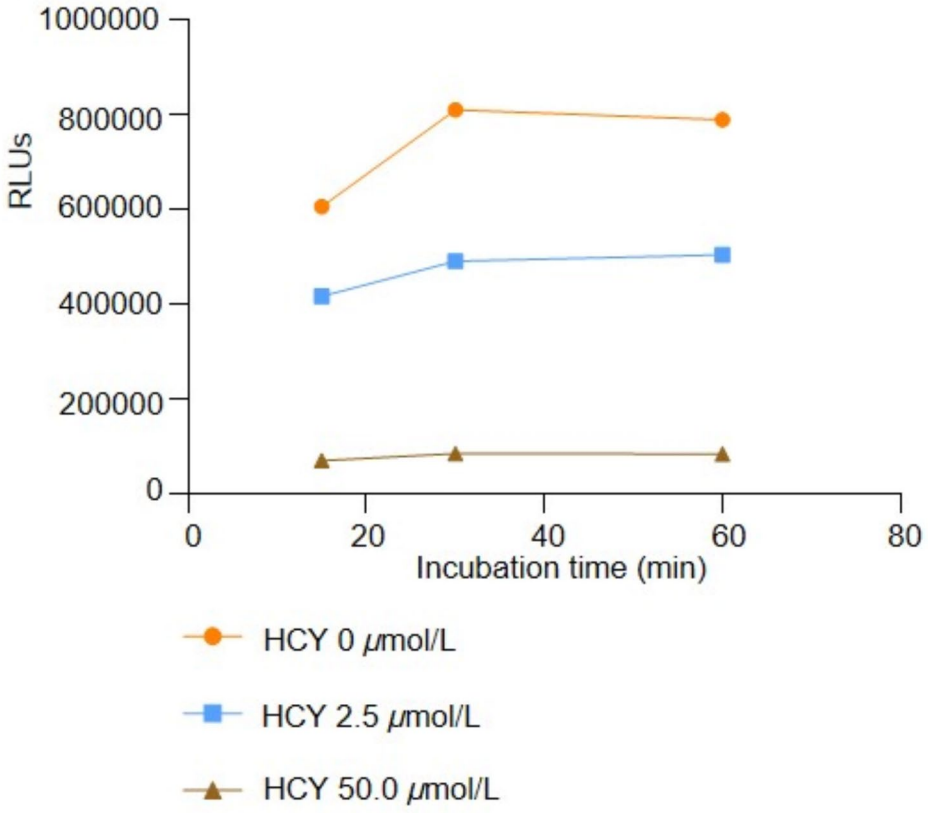



**Fig. 3** Optimization of incubation time

**Table 4** Reaction flow and reaction parameters

| Reaction step | Contents | parameters |
|---|---|---|
| Step 1 | Add calibrators, samples, quality controls | 30 μL |
| Step 2 | Adding a reducing agent | 15 μL |
| Step 3 | Mix well and react in a warm bath | 37℃, 5 min |
| Step 4 | Add enzyme | 15 μL |
| Step 5 | Magnetized particulate reagent | 30 μL |
| Step 6 | Addition of anti-reagents | 30 μL |
| Step 7 | Mix well and react in a warm bath | 37℃, 30 min |
| Step 8 | Add cleaning solution for chemiluminescence system and mix well. | 300 μL |
| Step 9 | Repeat step 8 for magnetic separation of the reactants | 3 times |
| Step 10 | Addition of chemiluminescent substrate solution for fully automated immunoassay for luminescence detection. | 200 μL |

that HCY levels in men were slightly higher than those in the serum collected from women (Fig. 5).

## Conclusions

The forms of HCY present in plasma include reduced HCY, disulfide HCY, and mixed HCY [32]. Under normal conditions, the plasma contains very little free HCY, mainly in the form of protein [33]. Many factors, such as genetics, nutrition, or drugs, can affect the metabolism of cysteine in the body, resulting in hyperhomocysteinemia [34]. High levels of HCY are associated with many diseases [7], such as Alzheimer's disease [35], diabetes [36], cancer [37], neurological disorders [38], age-related macular degeneration [39], vascular disease [40], and hearing loss [41]. Commonly used methods for HCY detection include HPLC, LC-MS, fluorescence polarization immunoassay (FPLA), enzyme immunoassay (ELISA), and cyclic enzyme assays [14–23]. However, these methods have disadvantages, such as the need for specialized equipment and technicians and a long operation time, which cannot meet the needs of the clinical testing of individual and emergency samples [3]. Of note, magnetic particle-based chemiluminescence immunoassay represents a novel analytical technique that integrates magnetic separation, chemiluminescence, and immunoassay [24–29]. This technology optimally exploits the rapid and automated magnetic separation, high sensitivity of chemiluminescence technology, and specificity of immunoassays [24–29]. These characteristics contribute to the high specificity, sensitivity, speed, and simplicity of CLIA, which has consequently been widely adopted in disease diagnosis [24–29]. Therefore, this kit adopts advanced magnetic separation chemiluminescence immunoassay

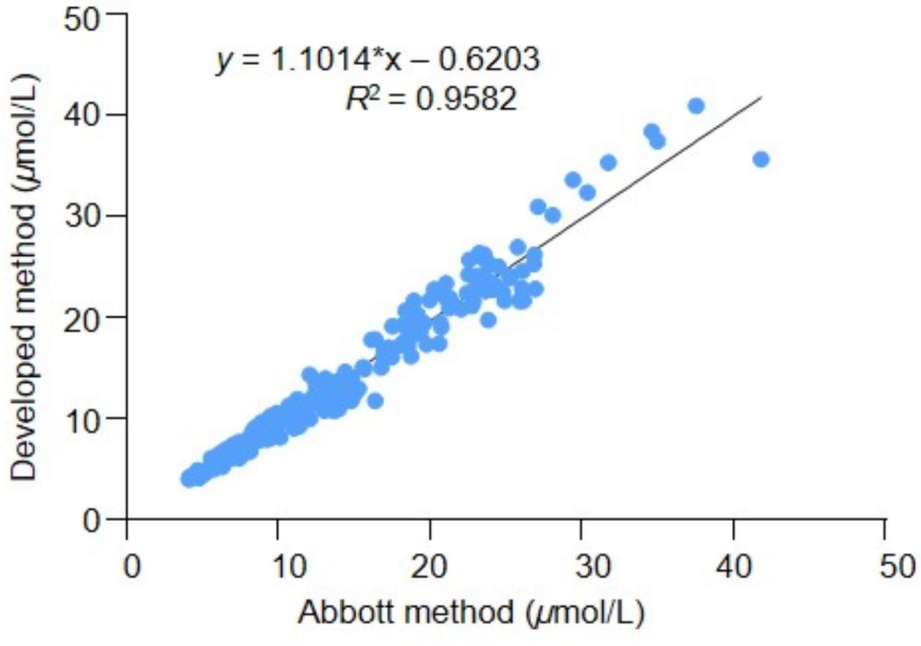



**Fig. 4** Comparison of the developed method and Abbott method

**Table 5** Distribution of clinical samples

| Populations | Conc.of HCY (μmol/L) | Cardiovascular diseases |
|---|---|---|
| Male | < 20 | 499 |
| | ≥ 20 | 50 |
| Female | < 20 | 431 |
| | ≥ 20 | 17 |

technology to detect HCY in blood. Through research on the process and reaction system, we determined the source of raw materials as well as quality standards and testing methods, formulated the production process, operation flow, quality standards and testing methods for each component, and established product standards and corresponding testing methods for the finished kit. In addition, we carried out a trial production of the product according to the established production process and quality control and evaluated the performance of the kit according to the determined reaction system and operation. The results showed that the production process of this product is stable, its quality is controllable, the reaction system is reasonable, and it meets the relevant technical, production, and clinical requirements. The results of clinical tests using this kit showed a good correlation with diseases and met clinical requirements. However, if this kit is to be used clinically in the future, a larger number of clinical test samples will be required for validation. Minimizing the variation in raw material lot helps maintain the performance of the kit.

Of note, serum HCY levels can be influenced by various factors, including age [42], sex [42], medication [28], and other variables [29]. The intake of methionine

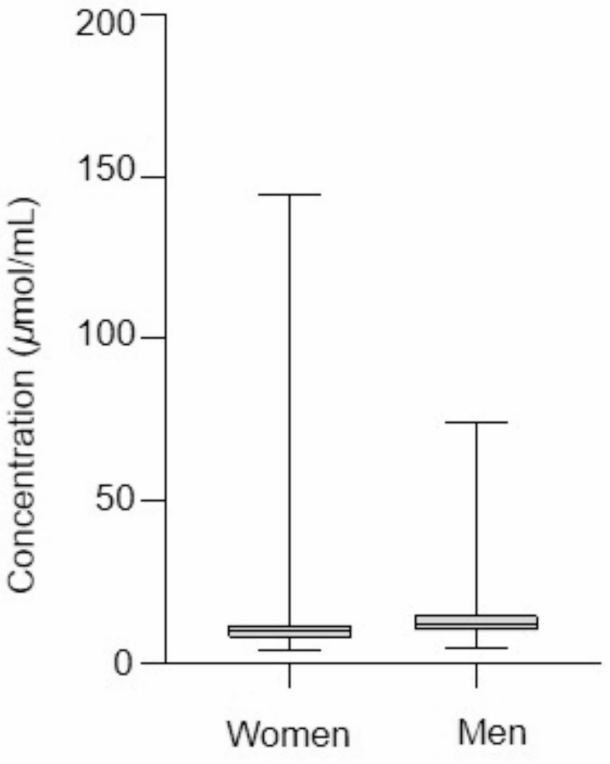



**Fig. 5** HCY concentration in women and men

and the supply of folate and vitamins B6 and B12 are among the dietary factors that influence HCY levels [43]. Genetic defects, polymorphisms, or mutations in several key enzymes can impair HCY metabolism, leading to

elevated HCY levels [29]. Additionally, some studies have observed a sex difference in plasma HCY levels [42]. Furthermore, HCY levels are higher in individuals aged 55 and above than in those below 55 years of age [44]. Based on these findings, we propose that the HCY reference value should be contextualized in clinical applications.

**Abbreviations**

| | |
|---|---|
| HCY | Homocysteine |
| HPLC | High Performance Liquid Chromatography |
| LC-MS | Liquid Chromatograph Mass Spectrometer |
| LoD | Limit of Detection |

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**Author contributions**
This project was conceived and supervised by YKL and XWQ. The method was designed by NS and XWQ (Fig. 1). Method performance validation, optimization (Figs. 2, 3 and 4), and all corresponding statistical analyses were performed by YKL and YRL. HCY concentrations of different patients (Fig. 5) were determined by RW. Patient sample collection was contributed by SNC. YKL, NS, and XWQ prepared the manuscript. All the authors reviewed the manuscript.

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**Data availability**
Data is provided within the manuscript information files. And, all other data and materials supporting the findings of this study are available from the corresponding authors upon reasonable request.

## Declarations

**Ethics approval**
This study was conducted in accordance with the Declaration of Helsinki. Prior to obtaining patient samples, requisite approval from the Affiliated Hospital of Jiangnan University and written informed consent were obtained from all patients.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare no competing interests.

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