A highly sensitive single-enzyme homocysteine assay

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A protocol for measuring the total plasma homocysteine (tHCY) concentration in very small samples using a selective, recombinant homocysteine α, γ-lyase (rHCYase) and a small portable fluorescence reader is described. The rHCYase produces hydrogen sulfide (H_2S) from tHCY without interference from physiological concentrations of Cys or other plasma components. H_2S is determined through reactivity with N,N-dibutyl phenylene diamine (DBPDA), which leads to the formation of a fluorescent chromophore. Only 5 μl of plasma/serum sample is required, which can be obtained from a finger prick, suggesting great potential for mass screening. The assay takes ~20 min.

INTRODUCTION

Numerous studies have reported that total plasma homocysteine (tHCY) plays a critical role in heart disease and stroke[1–5]. Lindner *et al.*[6] reported that hyperhomocysteinemia is a major risk factor of death due to cardiovascular disease in patients with end-stage renal disease (ESRD). Refsum *et al.*, Geisler *et al.* and Sun *et al.*[7–9] suggested that plasma tHCY could be used as a new tumor marker to monitor cancer patients during chemotherapy. Clarke *et al.*, Vafai *et al.* and Selhub *et al.*[10–12] reported that Alzheimer's disease patients had significant elevated plasma tHCY. Duan *et al.* and Allain *et al.*[13,14] reported the first direct evidence that homocysteine can sensitize dopaminergic neurons to dysfunction and death in models of Parkinson's disease. Wollesen *et al.* and Stehouwer *et al.*[15,16] reported that plasma tHCY levels were higher in diabetes. Vollset *et al.*[17] reported that elevated tHCY concentration is associated with common pregnancy complications and adverse pregnancy outcomes. Righetti *et al.*[18] demonstrated that significant lowering of tHCY in ESRD patients lowered the incidence of cardiovascular events, indicating a causal role for tHCY.

A number of plasma tHCY assays have been developed. High-performance liquid chromatography (HPLC) methods, the first to be developed[19–23], are accurate and have been used as the standard for tHCY. Disadvantages of HPLC methods for clinical application include the limited number of samples that can be processed and the fact that appropriate HPLC instruments and skilled operators may not be present in common clinical laboratories. Gas chromatography/mass spectrometry also requires highly specialized equipment and trained operators[20]. Shipchandler *et al.*, Frantzen *et al.* and Nexo *et al.*[24–26] have developed fluorescence polarization immunoassays for the detection of tHCY. These assays have good precision and little cross-reactivity toward Cys and Met. However, these methods have limited throughput and either involve numerous experimental steps or can only be implemented with very specialized instrumentation[27]. Recently, a selective chemical method to detect homocysteine has been developed. However, this method appears to require very large amounts of plasma (0.5 ml), which makes the assay impractical when a patient's plasma is used for multiple assays or for mass screening[28].

We have previously developed an enzymatic homocysteine assay, a simple single-enzyme tHCY assay based on a highly specific L-homocysteine α, γ-lyase (rHCYase) and a H_2S-specific chromogenic agent, N,N-dibutyl phenylene diamine (DBPDA)[29–31]. The assay relies on the rHCYase-catalyzed release from tHCY of hydrogen sulfide (H_2S), which upon reaction with DBPDA forms a chromophore, whose concentration can be measured either by a spectrophotometer or fluorometer[29]. The enzymatic HCY assay, based on the principles just described, has been applied to the Hitachi 912 Automatic Analyzer[31]. The FDA has cleared this assay in 2003 (510k no. K030754). A limitation of this approach is that it depends on a large and expensive piece of equipment.

In ANTICIPATED RESULTS, we describe a protocol to apply the enzymatic HCY assay using an inexpensive, portable fluorescence reader that can be used in small laboratories such as in a doctor's office. Only 5 μl plasma is required, which underscores the potential for mass screening. The assay is simple, accurate, precise and economic, and its throughput is ~100 tests per hour.

Experimental considerations

Negative controls for the homocysteine assay described here are blank tubes that contain all reagents but no plasma. Positive controls include tubes that have plasma with known amounts of homocysteine ranging from a low HCY control of 7–8 μM to a high HCY control of 24–26 μM. The assay is usually performed with two replicates when used clinically. Since the coefficient of variance (CV) is low, multiple replicates are usually not needed. In the assay described here, the fluorescence signal is high and is automatically quantified by the portable reader in micromoles of homocysteine per liter. The assay has great clinical potential for small laboratories, doctors' office laboratories and pharmacies for mass screening. The assay is also suitable for research laboratories studying homocysteine in animals. The protocol described here uses the Opulen fluorescence reader and is available through A/C Diagnostics LLC (San Diego, CA). However, any fluorescence reader set to EM 710 nm/EX 660 nm can be used.

Protocol overview

The first step of the assay (Reaction I), consists in using DTT to reduce any disulphide bonds present in the plasma. This procedure

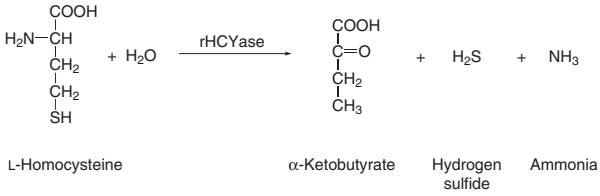

Figure 1 | Schematic representation of Reaction II: the formation of α-ketobutyrate, ammonia and hydrogen sulfide from L-homocysteine as catalyzed by rHCYase[28].

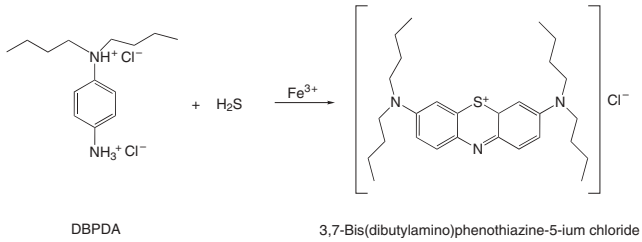

Figure 2 | Reaction of H_2S with DBPDA to form the fluorescent thiazine 3,7-bis(dibutyl amino)phenothiazine-5-ium chloride[28].

converts protein-bound L-homocystine to L-homocysteine (RS-SR to RSH + RSH).

rHCYase is then used to catalyze the conversion of L-homocysteine to α-ketobutyrate, ammonia and H_2S (Reaction II, see **Fig. 1**).

Finally, DBPDA chloride added to the reaction mixture reacts with H_2S to form a fluorescent thiazine (3,7-bis(dibutyl amino) phenothiazine-5-ium chloride) which can be detected quantitatively at $\lambda_{em} = 710$ nm/$\lambda_{ex} = 660$ nm (Reaction III, see **Fig. 2**).

MATERIALS

REAGENTS

- L-Homocystine (Sigma Chemical)
- L-Cys (Sigma Chemical)
- L-Met (Sigma Chemical)
- DTT (Sigma Chemical)
- Triton X-100 (Sigma Chemical)
- Citric acid (Sigma Chemical)
- Potassium ferricyanide (Sigma Chemical)
- 4-Fluoro-7-sulfobenzofurazan ammonium salt (Wako Fine Chemicals)
- N,N-dibutylaniline (Acros Organics)
- DBPDA (A/C Diagnostics)
- Recombinant rHCYase (A/C Diagnostics)
- Sodium phosphate monobasic anhydrous and sodium phosphate dibasic anhydrous (Fisher Scientific)
- Plasma matrix for calibrators and controls (Serologicals)
- Calibrators (containing tHCY levels) (A/C Diagnostics)
- Controls (containing tHCY levels) (A/C Diagnostics)

EQUIPMENT

- Fluorescence reader (Opulen)
- 96-Well plates (Fisher Scientific)
- Eppendorf tubes (Fisher Scientific)
- Pipettes (Fisher Scientific)
- Vacutainer tubes with K_2 EDTA (Becton Dickinson)

REAGENT SETUP

Preparation of solvent solutions (see **Box 1**, **Table 1**)
Preparation of reagents for reactions (see **Box 2**)
Preparation of calibrators and controls (see **Box 3**)

Reagent for Reaction I (see Protocol overview) Preparation of concentrated DTT stock solution (100×): dissolve 10 mg in 0.5 ml buffer (0.2 mM citric acid). To obtain 20 ml of working solution (1×), add 0.2 ml of DTT concentrated stock solution and 0.05 ml of surfactant into 19.75 ml buffer (40 mM sodium phosphate buffer pH 8.3), mix well.

Reagent for Reaction II Prepare 0.5 ml concentrated recombinant homocysteinase (rHCYase) stock solution (40×): dissolve 0.5 mg of the enzyme in 0.5 ml buffer (40 mM sodium phosphate buffer pH 8.3). To obtain 10 ml working solution (1×), add 0.25 ml of rHCYase concentrated stock solution into 9.75 ml buffer (40 mM sodium phosphate buffer pH 8.3).

DBPDA solution for Reaction III Prepare a 12.5 mM DBPDA solution in 1.5 N H_2SO_4.

Ferric solution for Reaction III Prepare 5 mM $K_3Fe(CN)_6$ (potassium ferricyanide) solution in 10 mM sodium phosphate buffer, pH 7.6.

EQUIPMENT SETUP

Fluorescence reader See **Supplementary Methods** online for the Operation Guide for the A/C Diagnostics fluorescence reader.

PROCEDURE

Plasma collection and storage ● TIMING 1 h

1| To determine baseline homocysteine levels, blood donors fasted for 12 h[23–27,29–31]. Collect blood into Vacutainer containing K_2 EDTA.

2| Separate plasma within 1 h by centrifugation at 300*g* for 10 min at 4 °C and collect into sterile tubes.

■ **PAUSE POINT** Plasma tHCY is stable for 1 month, 6 months and 2 years at 4 °C, −20 °C and −80 °C, respectively.

BOX 1 | PREPARATION OF SOLVENT SOLUTIONS

Preparation of Buffer I (10 mM sodium phosphate buffer, pH 8.3): add 98 ml of 0.1 M sodium phosphate dibasic and 2 ml of 0.1 M sodium phosphate monobasic into 900 ml ddH_2O to obtain Buffer I.
Preparation of Buffer II (0.1 mM pyridoxal 5-phosphate): dissolve 2.34 mg pyridoxal 5-phosphate in 100 ml of 10 mM sodium phosphate buffer (pH 8.3) to obtain Buffer II.
Preparation of solution for Reagent III (1.5 N H_2SO_4): add 10 ml of 18 N concentrated sulfuric acid into 110 ml ddH_2O to obtain 1.5 N H_2SO_4.
Preparation of solution for Reagent IV (10 mM sodium phosphate buffer, pH 7.6): add 8.45 ml of 0.1 M sodium phosphate dibasic and 1.55 ml of 0.1 M sodium phosphate monobasic into 90 ml ddH_2O to obtain 10 mM sodium phosphate buffer, pH 7.6.

TABLE 1 | Calibrators (S1–3) and controls (L and H) are prepared by spiking $[L\text{-HCY}]_2$ into the plasma matrix according to the volume ratios in the table.

	Volume of plasma (%)	Spiked 10% volume of $[L\text{-HCY}]_2$ (μM)	Values of L-HCY (μM) measured by HPLC
Calibrators			
S-1	90	0	3.2
S-2	90	50	15.6
S-3	90	200	43.8
Controls			
C-L	90	25	7.8
C-H	90	100	25.4

HCY, homocysteine; HPLC, high-performance liquid chromatography.

Setup of the fluorescence reader for testing ● TIMING 10 min

3| In the case of the Opulen portable fluorescence reader, set up by performing the following steps: (i) Turn on the reader. (ii) Select 'Settings'. (iii) Select 'STDs' and hit Enter. One can set up up to 10 STDs with a minimum of three STDs (STD0, STD1, STD2 … through STD9), which means you can set 10 different groups of standards (calibrators). Input the concentrations of the calibrators as calculated in Step 3. (iv) Select 'Printer' and hit Enter. Chose to either print or not print. (v) Select 'Trigger'. Choose Trigger by the either closing the lid or hitting the Enter key to start the reading process.

Reaction I ● TIMING 1 h

4| The assay can be performed either in a 96-well microplate or in 0.5 ml Eppendorf tubes or 0.5 ml PCR tubes. The conditions for the enzymatic HCY assay on the portable fluorescence reader are shown in **Table 2**. Add 5 μl of each sample/calibrator/control into one well of a 96-well plate or in a tube.

5| Add 165 μl of Reagent I, incubate at room temperature (24–28 °C) for 60 min or at 37 °C for 30 min.

Reaction II ● TIMING 5 min

6| Add 30 μl of Reagent II, incubate at room temperature (24–28 °C) for 5 min.

Reaction III ● TIMING 15 min

7| Add 50 μl Reagent III and let stand at room temperature for 5 min.

8| Add 30 μl Reagent IV and let stand at room temperature for 10 min.

Results read by portable fluorescence reader

9| If the assay is carried in a 96-well plate, transfer the solution to 0.5 ml tubes.

10| Read fluorescence at EM 710 nm/EX 660 nm and both the fluorescence results and the HCY value will be reported directly by the fluorescence reader.

BOX 2 | PREPARATION OF REAGENTS FOR REACTIONS

Reagent I for Reaction I

Preparation of DTT stock solution: dissolve 10 mg DTT in 0.65 ml of 0.2 mM sodium citrate (pH 3.0) to obtain a 100× DTT stock solution, which is stable for 5 days at 4 °C and 2 years at −80 °C.

Preparation of 20 ml Reagent I (reducing reagent) working solution: add 0.2 ml of DTT stock solution and 0.05 ml surfactant (Triton X100) into 19.75 ml Buffer I (10 mM sodium phosphate buffer, pH 8.3), mix well by vortex. The working solution is stable for 24 h at 4 °C.

Reagent II for Reaction II

Preparation of enzyme stock solution: dissolve 0.5 mg lyophilized recombinant homocysteinase (rHCYase) in 0.5 ml of Buffer I (10 mM sodium phosphate buffer, pH 8.3), to obtain a 40× enzyme stock solution, which is stable for 5 days at 4 °C and 2 years at −80 °C.

Preparation of 10 ml Reagent II (enzyme) working solution: add 0.25 ml of enzyme stock solution and 0.1 ml 100× Buffer II stock solution (0.1 mM pyridoxal 5-phosphate in10 mM sodium phosphate buffer, pH 8.3) into 9.65 ml Buffer I (10 mM sodium phosphate buffer, pH 8.3), mix well. The working solution is stable for 24 h at 4 °C.

Reagent III and IV for Reaction III

Preparation of Reagent III: dissolve 22 mg of DBPDA in 6 ml 1.5 N H_2SO_4, which is stable for 2 years at 4 °C.

Preparation of Reagent IV: dissolve 6.6 mg of $K_3Fe(CN)_6$ in 4 ml 10 mM sodium phosphate buffer, pH 7.6, which is stable for 2 years at 4 °C.

BOX 3 | PREPARATION OF CALIBRATORS AND CONTROLS

Plasma samples containing different concentrations of homocysteine are used for calibrators (S1, S2, S3) and controls (C-L, C-H) (**Table 1**). The plasma matrix for calibrators and controls was purchased from Serologicals Corporation. The plasma was Seracon II, Xase, Deplipidated, Lot no. 02E1202. L-homocysteine ($[L\text{-HCY}]_2$) was purchased from Sigma-Aldrich (cat. no. H6010). $[L\text{-HCY}]_2$, in a 2 mM stock solution, is prepared by dissolving $[L\text{-HCY}]_2$ in phosphate buffered saline (PBS, pH 7.2) (Fisher, cat. no. 21-040-CV), vortexed for 24 h at room temperature, then diluted with PBS to obtain a working solutions of 25, 50, 100 and 200 μM.

Calibrators (S1–3) and controls (L and H) were prepared by spiking $[L\text{-HCY}]_2$ into the plasma matrix according to the volume ratios in **Table 1**. The ranges of the calibrators and controls: calibrator-1: 3–4 (μM), calibrator-2: 14–16 (μM), calibrator-3: 42–45 (μM); control-L: 7–9 (μM), control-H: 22–25 (μM).

The exact values of calibrators and controls are measured by HPLC for each lot and labeled in the Instruction Manual of the kit.

HPLC determination for homocysteine: the HPLC assay for L-HCY in plasma uses the SBD-F derivatization method as described by Dudman *et al.*[22] and Ubbink *et al.*[19]. A reversed-phase Supelcosil LC-18-DB column (particle size 5 μm, 25 cm × 4.8 mm) (Supelco) is used on a Hitachi L-6200A HPLC (Hitachi).

If controls continue to be unstable, blood from new donors will be obtained to make new controls.

The calibrators and controls are prepared ready to use in the assay. The calibrators and controls will be analyzed in the assay according to the protocols as the same way as the samples, and, after the reactions finished, they will be measured by the Fluorescent Reader first, and then the samples will be measured.

Setup of the fluorescence reader for testing ● TIMING 15 min

11| In the case of Opulen reader, perform the following steps: (i) Turn on the reader. (ii) Select 'Settings'. (iii) Select 'STDs'. Input the concentrations of the calibrators as calculated in Step 3. (iv) Select 'Printer'. (v) Chose to print. (vi) Select 'Trigger'. Choose Trigger by either closing the lid or hitting the Enter key. (vii) Select 'Testing', then hit the Enter key. (viii) Input group number, then hit the Enter key. (ix) Choose grade of gain, then hit the Enter key.

12| Test the blank by closing the lid.

13| Test the calibrators (at least three different calibrators, see above). Then press 'F' key and chose Regression, check the line and the RR value and save it.

14| Press ESC once and save, then (i) enter sample test step; (ii) test each sample and obtain the results both of fluorescence and HCY values.

? TROUBLESHOOTING

? TROUBLESHOOTING

The assay is straightforward and simple. With standard care, the performance of the assay will not result in problems. However, preparation and condition of the plasma is critical. Plasma should be prepared within 1 h of blood draw in order that red-cell homocysteine not be secreted and alter the true value. In addition, lipemic plasma needs to be centrifuged in order to clarify the plasma.

ANTICIPATED RESULTS

To illustrate the types of results that might be obtained using this assay, we describe the results of the following specific experiments: (i) determination of linearity, recovery rate and limitations of quantitation; (ii) precision; (iii) method comparison; (iv) comparison of plasma and serum samples and (v) specificity and interference of the assay.

Linearity, recovery rate and the limit of quantitation of the enzymatic HCY assay on the portable fluorescence reader

Ten tests for five plasma samples containing different levels of L-HCY (3.2–44.6 μM) were measured by the enzymatic HCY assay on the Opulen reader. The data are shown in **Figure 3**, and the linearity of the assay is 4.1–43.8 μM.

TABLE 2 | Conditions for the enzymatic HCY assay.

Reaction	Reagent	Conditions		
		Volume (μl)	Time (min)	Temperature
I	Sample/calibrator/control	5	—	Room temperature (20–25°) for 60 min or 37 °C for 30 min
	Reagent I	165	60	
II	Reagent II	30	5	Room temperature
III	Reagent III	50	5	Room temperature
	Reagent IV	30	10	Room temperature

The recovery rates of the enzymatic HCY assay on the Opulen reader over the whole dynamic range are described below. One sample with tHCY concentration 45.0 μM was serially diluted 2, 4 and 8 times with PBS. The samples were analyzed by the enzymatic HCY assay in 10 replicates in a single run. The dilution recovery rates of the average values were calculated (**Table 3**).

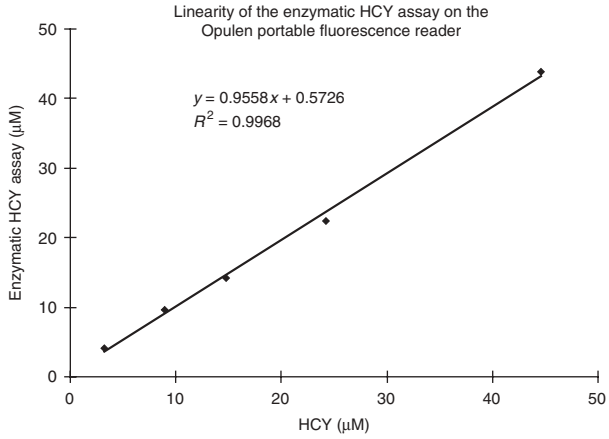

Figure 3 | Ten tests for five plasma samples containing different levels of L-HCY (3.2–44.6 μM) were measured by the enzymatic HCY assay on the Opulen reader. The linearity of the assay is 4.1–43.8 μM.

Precision of the enzymatic HCY assay on the portable fluorescence reader

Precision is determined by CV [CV(%) = SD/Average × 100]. SD is standard deviation among different tests for a sample. Precision increases as the CV decreases.

The precision of the enzymatic HCY assay on the Opulen reader was evaluated in a study based on NCCLS EP5-T2 guidelines 'Evaluation of Precision Performance of Clinical Chemistry Devices'[32]. In this study, the assay precision was determined after analyzing three plasma samples containing low, medium and high tHCY in parallel assays. The within-assay variation was determined from the results of 10 parallel analytical set-ups.

The within-assay precision of the enzymatic HCY assay on the Opulen reader was 4% for 8.1 μM tHCY; 5% for 14.9 μM tHCY; and 3% for 44.6 μM tHCY. Between-assay precision run over 10 days was 6.2% for 7.5 μM tHCY; 5.9% for 15.1 μM tHCY; and 4% for 46.0 μM tHCY. The CVs for within-assay and between-assay were ≤5 and 6.2%, respectively (**Table 4**). These precisions are within ranges reported for currently used assays[33].

Comparison between the automated enzymatic HCY assay and the enzymatic HCY assay on the portable fluorescence reader

The comparison of the enzymatic HCY assay performed with the Opulen portable fluorescence reader to the enzymatic HCY assay performed with the automated Hitachi 912 analyzer was carried out on forty plasma samples[34].

Table 5 shows the parameters of the automatic enzymatic HCY assay on the Hitachi 912 analyzer. The first column represents each reaction; the second column represents each reagent; the third column is the position of each reagent in the analyzer; the fourth and fifth columns represent the application parameters (volume of the sample and reagent and reaction time).

In the enzymatic HCY assay, the end point is read by absorbance at OD 700 nm/660 nm.

In the enzymatic HCY assay, the end point is read at fluorescence excitation 660 nm/emission 710 nm.

Table 2 shows the parameters of the portable enzymatic HCY assay. The description of **Table 2** is as follows.

The assay is carried out in a 96-well plate or 0.5 ml Eppendorf tubes or in 0.5 ml PCR tubes. (i) Add 5 μl of either samples/calibrators/controls into one well of a 96-well plate or in a PCR tube (Thermowell tube) or an Eppendorf tube, separately. (ii) Add 165 μl of Reagent I; incubate at room temperature (24–28 °C) for 60 min or at 37 °C for 30 min. (iii) Add 30 μl of Reagent II; incubate at room temperature (24–28 °C) for 5 min. (iv) Add 50 μl Reagent III; let stand at room temperature for 5 min. (v) Add 30 μl Reagent IV and let stand at room temperature for 10 min. (vi) If the assay is carried in 96-well plates, transfer the solution to Thermowell tubes. (vii) The end point is read at fluorescence of EM 710 nm/EX 660 nm, and the HCY value is printed out by the reader.

The results demonstrate a reasonably good agreement between the data obtained with the portable reader versus the Hitachi 912. The correlation and regression analysis of the two sets of data yielded $y = 1.07 + 0.99$ with a correlation coefficient of $R^2 = 0.94$ (see **Fig. 4a**). The distribution of the difference versus mean of paired tHCY values are shown in a Bland–Altman plot[34] (**Fig. 4b**). The mean difference between the two assays was 1.95 μM. The samples, both at low and high concentrations of tHCY, agreed well (see **Fig. 4**).

Comparison of results obtained using plasma and serum samples

To compare tHCY data measured on plasma versus serum samples, 6 ml blood collected from each donor were

TABLE 3 | Recovery rate.

Dilution	Theoretical tHCY (μM)	Observed tHCY (μM)	% Recovery
Undiluted	45.0	45.0	100
2	22.5	23.5	104
4	11.3	10.6	94
8	5.6	5.1	91

TABLE 4 | Precision of the enzymatic HCY assay on the Opulen reader.

Assay	Sample 1	Sample 2	Sample 3
Within-assay precision			
Average tHCY (μM)	8.1	14.9	44.6
SD	0.3	0.78	1.33
CV (%)	4.0	5.0	3.0
Between-assay precision			
Average tHCY (μM)	7.5	15.1	46.0
SD	0.5	0.9	1.8
CV (%)	6.2	5.9	4.0

TABLE 5 | Parameters for the enzymatic HCY assay on the Hitachi 912 Automatic Analyzer which are input to the 'Application parameter' section of the analyzer to determine the protocols of the assay.

Reaction	Reagent	Position in Hitach 912	Hitachi 912 Application parameters	
			Volume (μl)	Reaction time (min)
I	Calibrator	Covered refrigerated inner ring of sample disk	30	—
	Control	Covered refrigerated inner ring of sample disk	30	—
	Sample	Outer ring and middle ring of sample disk	30	—
	Reagent I	Reagent Disk-1	150	1.5
II	Reagent II	Reagent Disk-2	30	3.5
III	Reagent III	Reagent Disk-2	50	5
	Reagent IV	Reagent Disk-1	30	5

immediately separated into three different vacutainers: one without anticoagulant, one containing K_2 — EDTA and one containing heparin. Plasma and serum were separated from whole blood within 1 h by centrifugation[20,21]. High HCY samples were prepared by spiked-in L-homocysteine. tHCY was measured by the enzymatic HCY assay on the portable fluorescence reader. The tHCY values in EDTA-plasma, heparin-plasma and serum were highly correlated (slopes ranging between 0.970 and 0.997 and $R^2 \geq 0.990$, see **Fig. 5**).

Specificity and interference of the enzymatic HCY assay

To study the interference of L-Cys in the enzymatic HCY assay on the Opulen reader, a plasma sample with 11.4 μM tHCY was spiked with L-Cys to achieve L-Cys levels from 50 to 200 μM. These concentrations are within the normal range of L-Cys concentrations in plasma, which is 33–117 μM. The samples were analyzed in quadruplicate by the assay.

Percentage of cross-reactivity is calculated using the following equation:

$$\frac{\text{HCY (spiked sample} - \text{nonspiked sample)}}{\text{HCY (nonspiked sample)}} \times 100 = \%\ \text{cross-reactivity.}$$

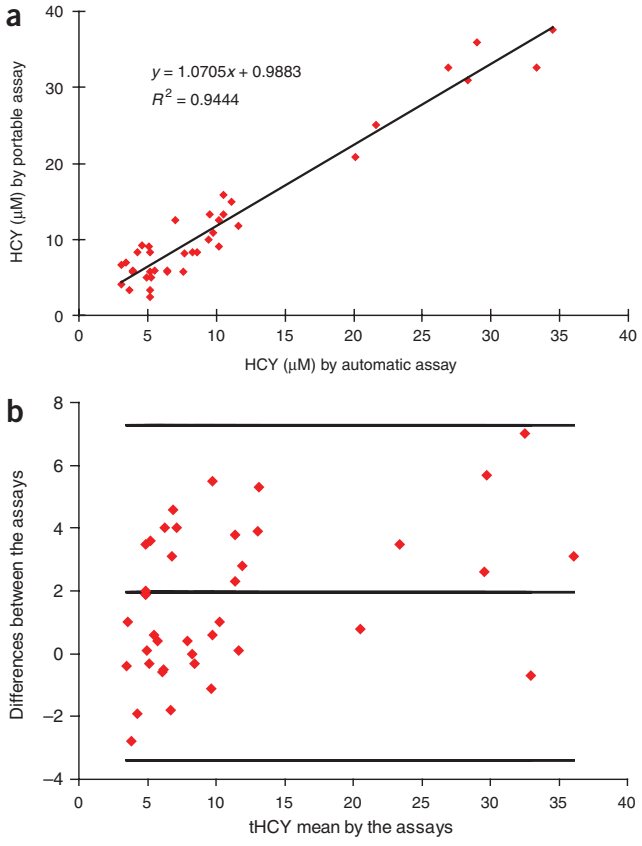

Figure 4 | Methods comparison. tHCY was measured in 40 samples both by the enzymatic HCY assay on the Opulen portable fluorescence reader and the enzymatic HCY assay on the Hitachi 912 Automatic Analyzer. (**a**) Linear regression analysis between the two sets of data with correlation coefficient. The correlation (R^2) was 0.94. (**b**) Bland–Altman plot, which shows the difference versus the mean of paired tHCY values between the enzymatic HCY assay on the Opulen reader and the enzymatic HCY assay on the Hitachi 912 (DF = difference in tHCY measured concentration, SD = standard deviation). The mean of the differences is 1.95 μM, and the tHCY, both at low and high concentrations, agree well.

Figure 6 shows Cys interference in the enzymatic HCY assay. The interference of Cys in the physiologic range (33–117 μM) in the enzymatic HCY assay is <2%.

The results demonstrate that L-Cys concentrations of up to 200 μM show <10% cross-reaction and, therefore, do not interfere with the measurement of tHCY by the assay (**Fig. 6**).

L-MET had no interference in the physical concentration of 0–200 μM. The interference of bilibrubin (0–1.6 mg ml^{-1}), lipid (0–5 mg ml^{-1}), hemoglobin (0–2.0 mg ml^{-1}), L-cystathionine (0–500 μM) and L-glutathionine (0–0.5 μM) were all <10% as well.

Conclusions

In summary, the enzymatic HCY assay on the portable fluorescence reader has acceptable within-assay and interassay precision (CV <5% and 8%, respectively). The assay is based on a highly specific rHCYase (ref. 35). The assay has sufficient linearity range (3.7–44.8 μM) for most clinical applications, although samples with very large tHCy concentrations may

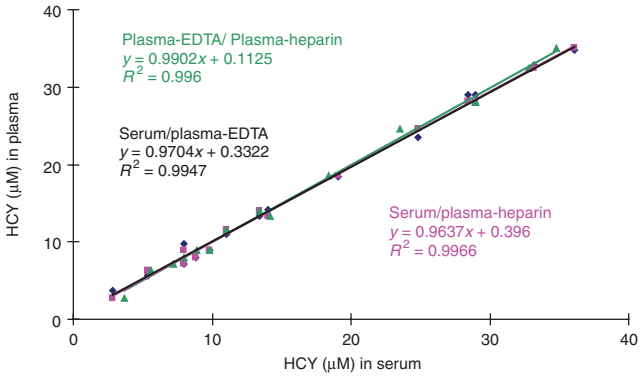

Figure 5 | Comparison of plasma and serum samples. Total HCY values measured in heparin-plasma, EDTA-plasma and serum samples obtained from 6 ml blood drawn from each donor were measured by the enzymatic HCY assay on the Opulen reader. Please note that high HCY samples were prepared by spiking in L-homocysteine into samples. The results showed tHCY values measured by this assay from the samples in EDTA-plasma, heparin-plasma and serum were highly correlated to each other (slopes ranging between 0.970 and 0.997 and $R^2 \geq 0.990$).

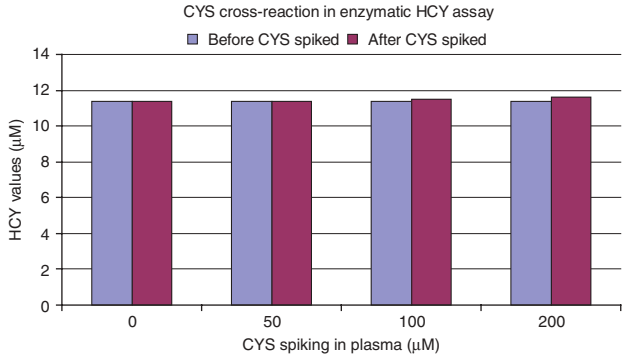

Figure 6 | Interference of Cys in the portable enzymatic HCY assay. Please see text for details.

have to be diluted. The portable enzymatic HCY assay method has a very high correlation with the enzymatic HCY assay on the Hitachi 912 automatic analyzer, which has been cleared by an FDA 510k.

Note: Supplementary information is available via the HTML version of this article.

COMPETING INTERESTS STATEMENT The authors declare competing financial interests (see the HTML version of this article for details).

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