Homogeneous Enzymatic Colorimetric Assay for Total Cysteine, *Qinghong Han, Mingxu Xu, Li Tang, Xinghua Sun, Nan Zhang, Xuezhong Tan, Xiuying Tan, Yuying Tan, and Robert M. Hoffman** (A/C Diagnostics LLC and AntiCancer, Inc., 7917 Ostrow St., San Diego, CA 92111; * author for correspondence: fax 858-268-4175, e-mail all@anticancer.com)

Patients with vascular disease have significantly higher concentrations of plasma total cysteine (tCYS) than do healthy individuals *(1–7)*. The present method is a new, rapid, and sensitive enzymatic colorimetric assay for tCYS in plasma samples and is homogeneous in that it avoids separation methods. The tCYS assay uses only the recombinant enzymes methionine α,γ-lyase (rMETase) and S-adenosylhomocysteine hydrolase (rSAHH) cloned from *Pseudomonas putida* and *Trichomonas vaginalis*, respectively. We have also developed enzymatic assay methods for total homocysteine (tHCY) *(8, 9)* and vitamin B_6 *(10)* in plasma that use the same analyte, H_2S, that is used for tCYS in the present report. Simultaneous assay of tHCY, vitamin B_6, and tCYS may be relevant to the study for the occurrence and prevalence of cardiovascular disease *(11)*.

The instrumentation included a Hitachi U-2000 Spectrophotometer (Hitachi, Ltd.) and Fl-1000 fluorescence spectrophotometer and a Hitachi HPLC (L-6200A Intelligent Pump) equipped with a Supelcosil LC-18DB column [25 cm × 4.8 mm (i.d.); particle size, 5 μm; Supelco].

The chemicals used were L-cysteine, D,L-homocysteine, L-methionine, adenosine (ADO), L-dithiothreitol, Triton X-100, pyridoxal 5-phosphate, and potassium ferricyanide and were purchased from Sigma Chemical Co. *N,N*-Dibutylphenylenediamine (DBPDA) was synthesized in our laboratory *(8, 9, 12)*. rMETase and SAHH were produced in our laboratory *(13, 14)*.

Four reagents were used for the assay. Reagent 1 contained 20 mmol/L potassium phosphate (pH 8.3), 150 mmol/L NaCl, 9 mg/L rSAHH, 2 mL/L Triton X-100, 1.0 mmol/L L-dithiothreitol, and 100 μmol/L ADO. Reagent 2 contained rMETase (1.08 g/L protein) in 20 mmol/L potassium phosphate (pH 7.2). Reagent 3 contained 40 mmol/L DBPDA in 3 mol/L HCl. Reagent 4 contained 15 mmol/L potassium ferricyanide in 20 mmol/L potassium phosphate (pH 7.2).

Whole blood was collected in evacuated blood-collection tubes without additive or containing EDTA, heparin, or citrate as anticoagulants. Centrifugation was carried out as soon as possible at 2000*g* for 10 min. The plasma was collected and stored at −70 °C until analysis.

The principle of the assay is as follows: In step 1, samples are reduced by L-dithiothreitol to generate free reduced CYS and HCY. Simultaneous use of rSAHH with excess ADO converts the reduced HCY to *S*-adenosylhomocysteine (SAH).

$$\text{Step 1: Reduced HCY + ADO} \xrightarrow{\text{SAHH}} \text{SAH + } H_2O$$

In step 2, rMETase is added to generate H_2S from tCYS:

$$\text{Step 2: Reduced CYS} \xrightarrow{\text{rMETase}} \text{Pyruvate + } H_2S + NH_3$$

In step 3, H_2S combines with DBPDA to form a compound that is fluorescent and has ultraviolet absorbance. For this assay, the absorbance is read at 675 nm.

$$\text{Step 3: DBPDA + } H_2S \xrightarrow{K_3Fe(CN)_6}$$

3,7-bis(dibutylamino)-phenothiazine-5′-ium chloride

The tCYS enzymatic assay protocol was as follows:

Step 1. To remove the tHCY in samples, 20 μL of calibrator or plasma sample and 980 μL of reagent 1 were added. This reaction was carried out at 37 °C for 30 min. This reaction was essential for release of HCY from disulfide linkages in plasma proteins and its removal to form *S*-adenosylhomocysteine.

Step 2. For production of H_2S from tCYS by rMETase, reagent 2 (10 μL) was added to the tubes, which were then vortex-mixed and incubated at 37 °C for 10 min.

Step 3. For the chromogenic reaction, the enzymatic reaction was stopped by addition of 50 μL of reagent 3 followed by 50 μL of reagent 4. The chromogenic reaction was carried out at 37 °C for 10 min. tCYS was measured by its absorbance at 675 nm. Calibration was performed with use of calibrators before the first run every day.

The HPLC assay for tCYS and tHCY used the ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate derivatization method as described by Ubbink et al. *(15)* and Dudman et al. *(16)* with some modifications. Plasma samples were reduced with tri-*n*-butylphosphine and then derivatized with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate. tCYS was measured by HPLC with a reversed-phase Supelcosil LC-18DB column at room temperature.

Within-run imprecision was calculated by measuring tCYS in duplicate 20 times. The imprecision (CV) was 4.3% at a mean tCYS concentration of 189.1 μmol/L and 4.5% at a mean tCYS concentration of 283.4 μmol/L. Between-run imprecision was based on results from 20 successive analyses performed over 20 days. The imprecision (CV) was 4.9% and 4.7% at mean tCYS concentrations of 189.1 and 283.4 μmol/L, respectively.

To examine the linearity of the calibration curve, we measured five different L-CYS solutions (10, 62.5, 125, 250, and 500 μmol/L) with the tCYS enzymatic assay. The calibration curve was linear between 10 and 500 μmol/L, and linear regression analysis gave a relationship of: $y = 0.0005x + 0.0008$ μmol/L ($r^2 = 0.9973$).

The analytical recoveries for crystalline L-CYS added to pooled human plasma were determined. L-CYS (50–200 μmol/L) was added to the plasma samples containing 153 μmol/L endogenous L-CYS. The mean recovery was ~99%.

The tCYS concentrations in 40 different human plasma samples were measured with the tCYS enzymatic assay and a conventional HPLC method. As shown in Fig. 1, the methods showed excellent agreement. Deming regression analysis *(17)* comparing the enzymatic assay (x) and the HPLC method (y) yielded the following: $y = 1.0054x - 0.6716$ ($r^2 = 0.93$; n = 40). The mean difference between the methods, as determined by Bland–Altman difference plot *(18)* of paired means of the enzymatic and HPLC tCYS assays, was 0.645 μmol/L.

We tested the interference in the enzymatic tCYS assay by adding L-HCY, L-methionine, L-cystathionine, and cysteinylglycine to plasma samples. This experiment tested the cross-reactivity of rMETase to potentially interfering compounds (Table 1). No interference by HCY up to 200 μmol/L, L-methionine up to 100 μmol/L, L-cystathionine up to 130 μmol/L, and cysteinylglycine up to 200 μmol/L was observed. This is expected because of the high specificity of rMETase *(19)*. To test whether the assay was influenced by hemolysis, bilirubin, and triglycerides, we added different concentrations of hemoglobin, bilirubin, and triglycerides to the plasma preparation. The assay was unaffected by hemoglobin up to 1.0 g/L, bilirubin up to 0.82 g/L, and triglycerides up to 5.0 g/L.

Table 1. Interference in the enzymatic tCYS assay.

Analyte added	tCYS, μmol/L	Interference,[a] %
L-HCY, μmol/L		
0	147.0	
50	139.5	−5.1
100	153.7	4.6
200	152.0	3.4
L-Methionine, μmol/L		
0	147.0	
50	149.5	1.7
100	140.3	−4.6
200	133.7	−9.0
L-Cystathionine, μmol/L		
0	260.3	
32.5	250.2	−3.8
65.0	257.0	−1.3
130	252.2	−3.8
Cysteinylglycine, μmol/L		
0	203.2	
50	207.4	2.1
100	201.7	−0.7
200	206.4	1.6

[a] [(Sample with added analyte − sample without added analyte)/sample without added analyte] × 100%.

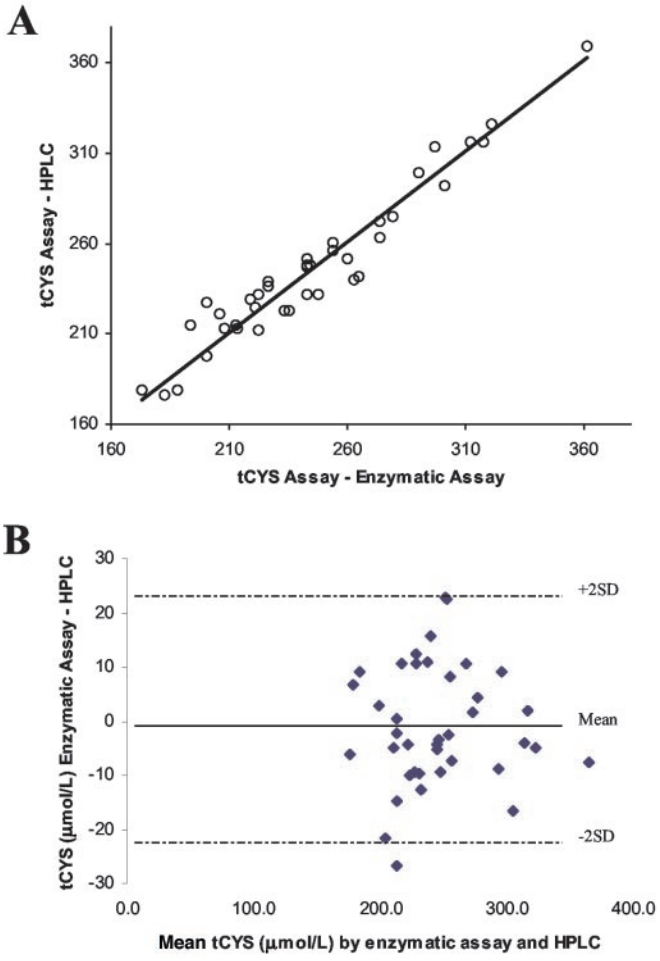

Fig. 1. Comparison of the enzymatic and HPLC tCYS assays.

(*A*), correlation between the enzymatic and HPLC tCYS assays by Deming regression analysis *(17)*. The results are expressed in μmol/L for the Deming regression analysis: $y = 1.0054x - 0.6716$ μmol/L ($r^2 = 0.93$; n = 40). (*B*), Bland–Altman plot of the difference in tCYS values between the enzymatic and HPLC tCYS assays as a function of their mean value ± 2 SD *(18)*. The mean difference between the methods was 0.0645 μmol/L.

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Breast Cancer Susceptibility Gene mRNAs Quantified by Microarrays with Electrochemical Detection, *Hong Xie, Yuan Hong Yu, Fang Xie, Yuan Zhi Lao, and Zhiqiang Gao** (Institute of Bioengineering and Nanotechnology, Singapore, Republic of Singapore; * address correspondence to this author at: Institute of Bioengineering and Nanotechnology, 51 Science Park Road, Singapore 117586, Republic of Singapore; fax 65-6874-9341, e-mail zqgao@ibn.a-star.edu.sg)

Abnormalities in the expression of specific genes have been linked to a large and increasing number of diseases. Quantification of gene expression is a promising basis for early diagnosis, but analysis at the mRNA level has shown to be difficult because of the limited sensitivity of existing nucleic-acid-detection techniques. The most commonly used methods for quantification of gene expression include Northern blotting *(1)*, ribonuclease protection assays (RPAs) *(2)*, and reverse transcription-polymerase chain reaction (RT-PCR) *(3, 4)*. The main limitation of the first two techniques is their relatively low sensitivity. RT-PCR can theoretically amplify a single nucleic acid molecule millions of times, but optimization of primer sets prolongs the assay time, and different genes in a starting mRNA mixture may not be present in the same amounts in the final RT-PCR products because of selective and nonlinear target amplification *(5)*. These limitations affect the precision and quality of the resulting data and often provide distorted information on gene expression. Sensitive, reliable gene detection is one of the challenges in molecular diagnostics.

Electrochemical detection provides a simple, accurate, and inexpensive platform for molecular diagnostics. Despite the enormous progress made in electrochemical nucleic acid biosensor research in the past 5 years, to be one step closer to commercialization, this research must overcome several important hurdles. The first is validation of the biosensor results on a statistically large population of real samples rather than the commonly reported relatively short synthetic oligonucleotides *(6)*. Another challenge is to multiplex the electrochemical biosensors into useful sensor arrays. Typically, arrays of 30–100 sensors are needed for diagnostic purposes. For example, breast cancer screening requires the testing of 20–30 cancer susceptibility genes plus positive and negative controls *(7)*. Of the many proposed electrochemical detection schemes, only a few attempts have been made to detect gene expression at the mRNA level *(8)*.

Here we describe an ultrasensitive method for direct detection of expression of breast cancer susceptibility genes in human breast tissues on an 8 × 8 sensor array. The human breast tissues were stored in liquid nitrogen immediately after surgery until mRNA extraction. Tissues were mechanically homogenized, and mRNA was extracted by use of a Dynabeads® mRNA DIRECT™ Kit (Dynal ASA) according to the manufacturer's protocol. Labeling of the mRNA was carried out with cisplatin-coupled biotin conjugates (Biotin-Chem-Link™; Roche Diagnostics) according to the manufacturer's recommended procedure. All solutions were treated with diethyl pyrocarbonate, and surfaces were decontaminated with RNaseZap (Ambion).

To fabricate the sensor array, we evaporated a titanium adhesion layer (25–50Å) on a glass slide by use of an electron beam, followed by 2500–3000 Å of gold. A patterned 1-mm thick adhesive spacing/insulating layer with a screen-printed Ag/AgCl layer and a hydrophobic layer were assembled on the top of the slide (Fig. 1 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol50/issue7/). The diameter of the individual sensor was 2.0 mm, and that of the top hydrophobic pattern was 4 mm. We immobilized 24mer oligonucleotides on each individual sensor surface, which served as capture probes, as described previously *(9)*. The sequences of the captured probes were complementary to the sequence of each gene in a region specific to that gene where no mutation has been reported.

We examined breast cancer susceptibility genes, i.e., tumor protein p53 (*TP53*; 1182 bp), heat-shock protein 90 (*HSP90*; 1632 bp), breast cancer gene 1 (*BRCA1*; 5592 bp), and Histone H4 (*His4*; 312 bp), plus a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; 1008 bp), covering both high-/low-copy number and long/short genes. Hybridization was carried out in a 55 °C water bath in a buffer containing 10 mmol/L Tris-HCl, 1.0 mmol/L EDTA, and 0.10 mol/L NaCl for 30 min. Before the hybridization, nucleic acid samples were denatured at 95 (cDNA) and 70 °C (mRNA) for 10 min and cooled in an ice bath before being added to the sensor array. After hybridization, the sensor array was exposed to 2.5-μL aliquots of 5.0 g/L glucose oxidase-avidin at room temperature for 30 min and then to 2.5-μL aliquots of 5.0 g/L redox polymer, poly(vinylimidazole-co-acrylamide) partially imidazole-complexed with Os(2,2′-bipyridine)$_2$(imidazole) for 10 min. Synthesis of the redox polymer has been described elsewhere *(10)*. The formation of a mixed micro three-dimensional mRNA + glucose oxidase–avidin/redox polymer bilayer allowed elec-