# Enzymatic assay for total plasma Cys

Qinghong Han & Robert M Hoffman

AntiCancer, Inc., 7917 Ostrow Street, San Diego, CA 92111, USA. Correspondence should be addressed to R.M.H. (all@anticancer.com).

Published online 23 October 2008; doi:10.1038/nprot.2008.178

**Patients with vascular disease and end-stage renal disease have significantly higher concentrations of plasma total Cys (tCys) than do healthy individuals. Described here is a nonradioactive, precise, 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. The rSAHH traps homocysteine and the rMETase thereby produces $H_2S$ exclusively from Cys. The reaction product, $H_2S$, is measured colorimetrically following its reaction with N,N-dibutylphenylenediamine (DBPDA). The procedure takes 3–4 h to complete.**



## INTRODUCTION

Patients with vascular and end-stage renal disease have significantly higher concentrations of plasma total Cys (tCys) than do healthy individuals[1–7]. The present method, which we have recently developed[8], is a rapid, precise and sensitive enzymatic colorimetric assay for tCys in plasma or serum samples and is homogeneous in that it does not require chromatographic or other 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. The rSAHH traps homocysteine (HCY) and the rMETase thereby produces $H_2S$ exclusively from Cys. We have also developed enzymatic assay methods for total HCY (tHCY)[9] and vitamin $B_6$ (ref. 10) in plasma that use the same analyte, $H_2S$, that is used for tCys in the present article. $H_2S$ is combined with N,N-dibutylphenylenediamine (DBPDA) to form an absorbent and fluorometric chromosphore. Simultaneous assay of tHCY[11,12], vitamin $B_6$, and tCys may be relevant to the study for the occurrence and prevalence of cardiovascular and other diseases[13].

In addition to plasma and serum, the method we describe can be directly used for assay of tCys in urine and other biological fluids (Q.H. and R.M.H., unpublished data), and thus we have used a wide range of concentrations (25–400 μM). The normal range for plasma or serum Cys is 250–275 μM[13].

### Principle of the assay

In Step 1, samples are reduced by DTT to generate free reduced L-Cys and HCY. Simultaneous use of rSAHH with excess Ado converts the reduced HCY to *S*-adenosylhomocysteine, and eliminates interference by HCY. In Step 2, rMETase is added to generate $H_2S$ from Cys[14] and in Step 3 $H_2S$ combines with DBPDA to form an absorbent/fluorescent compound. For this assay, the absorbance is read at 675 nm (**Fig. 1**).

The major limitation of our enzymatic assay is that it is an optical method. Therefore, highly lipimic samples must be cleared of opaque material by centifugation before use. HPLC has been the previous method of choice to measure Cys[15,16]. Although HPLC can use opaque samples, it suffers from the need of expensive equipment and for highly trained technicians.

Negative controls use phosphate buffer (10 mM, pH 7.6). Positive controls are 350 μM Cys for the high control and 200 μM Cys for the low control. The standard curve uses a range from 25 to 400 μM Cys using potassium phosphate buffer pH 8.3. A minimum of three replicates should be used if possible for each sample or standard.

## MATERIALS

### REAGENTS

- Potassium phosphate monobasic (Sigma-Aldrich)
- Potassium phosphate dibasic (Sigma-Aldrich)
- EDTA sodium salt (Sigma-Aldrich)
- L-cystine (Cys-Cys) (Sigma-Aldrich)
- DL-HCY (Sigma-Aldrich)
- L-Met (Sigma-Aldrich)
- Ado, L-DTT (Sigma-Aldrich)
- Triton X-100, pyridoxal 5-phosphate (PLP) (Sigma-Aldrich)
- Potassium ferricyanide (Sigma-Aldrich)
- DBPDA (A/C Diagnostics)[17]
- rMETase (AntiCancer)[18]
- rSAHH (A/C Diagnostics)[19]

### EQUIPMENT

- Multichannel pipettes (Transferpette)
- 96-well microplates (Corning)
- Microplate reader (Sunrise Tecan Reader; Trading AG) which has a 675-nm absorbance filter
- Multiplate shaker-IEMS Incubator/Shaker (Thermo Scientific)

### REAGENT SETUP

**Reagent 1** Contains 20 mM potassium phosphate, pH 8.3, 150 mM NaCl, 9 μg/ml SAHH, 0.2% (vol/vol) Triton X-100, 1.0 mM DTT and 100 μM adenosine.

**Reagent 2** Contains 20 mM potassium phosphate, pH 8.3, and 0.375 mg/ml rMETase. ▲ CRITICAL Make up Reagents 1 and 2 fresh each day, especially DTT in Reagent 1, needed to reduce the disulfide forms of Cys and HCY.

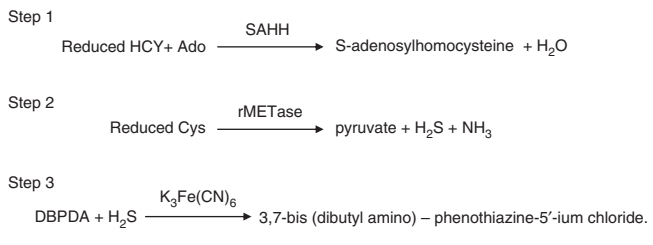


**Figure 1** | Scheme of the assay.

**Chromogen I (DBPDA)** Add 25 mM DBPDA in 3.0 M HCl. The solution should be stable for up to 2 months at room temperature.
**Chromogen II** 10 mM potassium ferricyanide (Sigma). The solution should be stable for up to 2 months at room temperature.
**L-Cystine (Cys-Cys) standards** Prepare 10 mM L-Cys stock solution by dissolving 12.12 mg in 10 ml of 20 mM potassium phosphate pH 8.3, and then make dilutions to create standard concentrations ranging from 25 to 400 μM Cys as the assay calibrators (25, 50, 100, 200, 300 and 400 μM) (**Fig. 2**).
**Low control and high control** Plasma used for controls is obtained from healthy volunteers and divided into two plasma pools for spiking with low- and high-concentration Cys.

**EQUIPMENT SETUP**

**Microplate reader** Set up a 96-well multidetection plate reader to run endpoint measurement read at an absorbance wavelength of 675 nm. Check software and reader requirements for the correct Calibrators/Controls configurations.

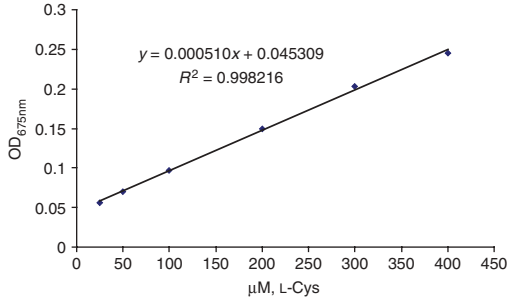


**Figure 2 |** Dilution linearity. Linearity was assessed by making serial dilutions of human plasma spiked with 267 μM Cys. The recovery of spiked Cys was 97.37%. Linearity was demonstrated by linear regression analysis with an *r* value >0.999.

## PROCEDURE

**1|** Aseptically collect blood samples by venipuncture. A minimum of 0.5 ml blood is collected asceptically into an EDTA venipuncture tube. Plasma is prepared by spinning at 1,000*g* for 10 min at room temperature.
**! CAUTION** For all blood and blood-derived samples, always observe precautions: handle all biological samples as a potential source of pathogens, use the appropriate protective attire (lab coats, safety glasses, latex gloves and other measures) and dispose of all biohazardous materials properly.

**2|** If plasma contains visible particulate matter, spin down utilizing high-speed centrifugation (8,000*g* for 10 min at 2–8 °C) before assay run.

**3|** Plasma may be stored up to a week at 2–8 °C. If a further delay in testing is needed store frozen at −70 °C in a freezer. Avoid multiple freeze/thaw of patient samples.

**4|** Transfer 10 μl of samples including calibrators and low and high controls to each well of a 96-well plate using pipette (20 μl) in triplicate (see **Fig. 3** for an example for analyzing 22 samples).

**5|** Add 200 μl of Reagent 1 to each well, use a multiple pipette.

**6|** Shake and incubate at 37 °C for 30 min.

**7|** Add 30 μl of Reagent 2 to each well of the reaction plate.

**8|** Shake and incubate at 37 °C for 5 min.

**9|** Add 25 μl chromogen I and 15 μl chromogen II to each well of the plate.

**10|** Shake and incubate reaction plate at room temperature for 5 min.

**11|** Read the plate at 675 nm.

**12|** The tCys values are calculated from the calibration curve.
**▲ CRITICAL STEP** The addition of all reagents in the assay must be consistent. It is suggested that pipetting should be in the same order from well to well, and at the same rate.
**? TROUBLESHOOTING**

**● TIMING**
Step 1: 10 min; Step 2: 10 min; Step 3: 1 min; Step 4: 5 min; Step 5: 1 min; Step 6: 30 min; Step 7: 30 s; Step 8: 5 min; Step 9: 30 s; Step 10: 5 min; Step 11: 30 s

**? TROUBLESHOOTING**
Troubleshooting advice can be found in **Table 1**.

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | BL(C0) | BL(C0) | BL(C0) | LC | LC | LC | S7 | S7 | S7 | S15 | S15 | S15 |
| B | C1 | C1 | C1 | HC | HC | HC | S8 | S8 | S8 | S16 | S16 | S16 |
| C | C2 | C2 | C2 | S1 | S1 | S1 | S9 | S9 | S9 | S17 | S17 | S17 |
| D | C3 | C3 | C3 | S2 | S2 | S2 | S10 | S10 | S10 | S18 | S18 | S18 |
| E | C4 | C4 | C4 | S3 | S3 | S3 | S11 | S11 | S11 | S19 | S19 | S19 |
| F | C5 | C5 | C5 | S4 | S4 | S4 | S12 | S12 | S12 | S20 | S20 | S20 |
| G | C6 | C6 | C6 | S5 | S5 | S5 | S13 | S13 | S13 | S21 | S21 | S21 |
| H | R1 | R1 | R1 | S6 | S6 | S6 | S14 | S14 | S14 | S22 | S22 | S22 |

**Figure 3 |** 96-well microplate layout. Please see text for details. C1–C6 are six concentrations of Cys (25–400 μM), LC and HC are the low and high control (350 and 200 μM Cys), BL(C0) is phosphate buffer negative control, R1 is repeat samples and S1–S22 are the samples to be analyzed.

**TABLE 1** | Troubleshooting table.

| Problem | Potential cause | Solution |
|---|---|---|
| Large decrease in absorbance signal | Loss of enzyme activity | Maintain enzymes on ice |
| | | Check enzyme activity |
| | Reagent expired | Ensure DTT is added to buffer immediately before assay |
| Solution color fades rapidly | Chromogen I contaminated with Chromogen II or Chromogen II was added before Chromogen I | First add Chromogen I, then Chromogen II |
| Foaming observed in wells | Bubbles occurred during addition of sample and reagents | Avoid bubbles in all steps |



## ANTICIPATED RESULTS

Within-run imprecision can be calculated by measuring tCys in duplicate 20 times. In our hands, the imprecision (CV) was 4.3% at a mean tCys concentration of 189.1 μM and 4.5% at a mean tCys concentration of 283.4 μM. Between-run imprecision should be based on results from 20 successive analyses performed over 20 d. In our lab, the imprecision (CV) was found to be 4.9 and 4.7% at mean tCys concentrations of 189.1 and 283.4 μM, respectively[8]. For HPLC, the CVs were found to range from 0.9 to 3.4% for within-run and 1.5 to 6.1% for between-run CVs[16].

To examine the linearity of the calibration curve, five different L-Cys solutions (10, 62.5, 125, 250 and 500 μM) should be measured with the tCys enzymatic assay. In our hands, the calibration curve was linear between 10 and 500 μM, and linear regression analysis gave a relationship of: $y = 0.0005x + 0.0008$ μM ($r^2 = 0.9973$)[8].

To determine the analytical recoveries, crystalline L-Cys was added to pooled human plasma and the concentration determined as described in the PROCEDURE. In an experiment that we performed, L-Cys (50–200 μM) was added to the plasma samples containing 153 μM endogenous L-Cys and the mean recovery was ~97.37%[8].

To compare the tCys enzymatic assay with a conventional HPLC method, the tCys concentrations in 40 different human plasma samples were measured with both methods. As shown in **Figure 4**, the methods showed excellent agreement. Deming regression analysis[20] to compare 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 the Bland–Altman difference plot[21] of paired means of the enzymatic and HPLC tCys assays, was 0.645 μM[8].

Interference in the enzymatic tCys assay can be tested by adding L-HCY, L-Met, L-cystathionine and cysteinylglycine to plasma samples. This experiment tests the cross-reactivity of rMETase to potentially interfering compounds (**Table 2**).

**TABLE 2** | Interference in the enzymatic tCys assay.

| Compound added | tCys (μM) | Interference* (%) |
|---|---|---|
| *L-HCY* (μM) | | |
| 0 | 147.0 | |
| 50 | 139.5 | −5.1 |
| 100 | 153.7 | 4.6 |
| 200 | 152.0 | 3.4 |
| *L-Met* (μM) | | |
| 0 | 147.0 | |
| 50 | 149.5 | 1.7 |
| 100 | 140.3 | −4.6 |
| 200 | 133.7 | −9.0 |
| *L-Cystathionine* (μM) | | |
| 0 | 260.3 | −3.8 |
| 32.5 | 250.2 | −1.3 |
| 65.0 | 257.0 | −3.8 |
| 130 | 252.2 | |
| *Cysteinylglycine* (μM) | | |
| 0 | 203.2 | |
| 50 | 207.4 | 2.1 |
| 100 | 201.7 | −0.7 |
| 200 | 206.4 | 1.6 |

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

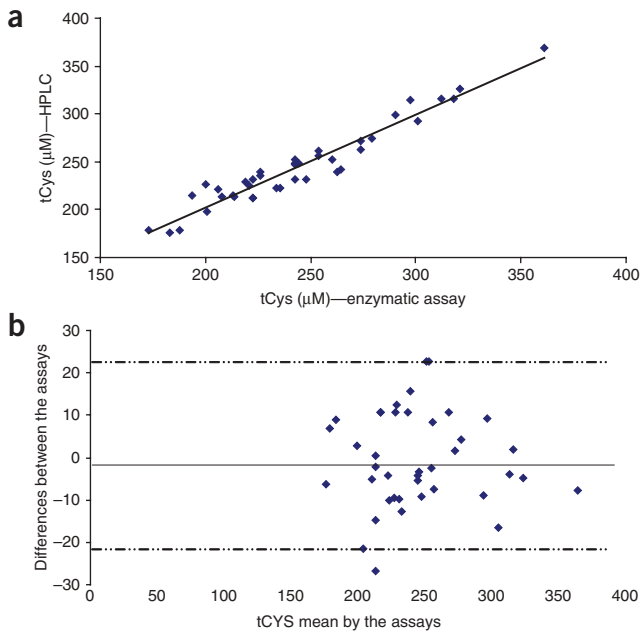


**Figure 4** | Comparison of the enzymatic and HPLC tCys assays. (**a**) Correlation between the enzymatic and HPLC tCys assays by Deming regression analysis[20]. The results are expressed in μM for the Deming regression analysis: $y = 1.0054x - 0.6716$ μM ($r^2 = 0.93$; $n = 40$). (**b**) Bland–Altman plot of the difference in tCys values between the enzymatic and HPLC tCys assays as a function of their mean value ± 2 s.d.[21]. The mean difference between the methods was 0.0645 μM.

In experiments performed in our laboratory, no interference by HCY up to 200 μM, L-Met up to 100 μM, L-cystathionine up to 130 μM, and cysteinylglycine up to 200 μM was observed. This is expected because of the high specificity of rMETase[21]. 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[8].

As stated in the INTRODUCTION, the normal, middle range for plasma or serum Cys is 250–275 μm/l. The normal values for Cys in the urine is 33.4 μmol/mmol creatinine for males and 28.3 for females (13). The levels of cystine (Cys) are markedly elevated in cystinuria from 320 to 1,420 μmol/mmol creatinine. Cystinuria is an autosomal recessive disorder characterized by impaired handling of Cys by the renal proximal tubules and intestines[22].

Published online at http://www.natureprotocols.com/
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

1. Nygard, O., Vollset, S.E., Refsum, H., Brattstrom, L. & Ueland, P.M. Total homocysteine and cardiovascular disease. *J. Intern. Med.* **246**, 425–454 (1999).
2. Ueland, P.M., Refsum, H., Beresford, S. & Vollset, S.E. The controversy over homocysteine and cardiovascular risk. *Am. J. Clin. Nutr.* **72**, 324–332 (2000).
3. McCully, K.S. Homocysteine and vascular disease. *Nat. Med.* **2**, 386–389 (1996).
4. Finkelstein, J.D. Methionine metabolism in mammals. *J. Nutr. Biochem.* **1**, 228–237 (1990).
5. Finkelstein, J.D. The regulation of homocysteine metabolism. In *Homocysteine Metabolism: from Basic Science to Clinical Medicine* (eds. Graham, I., Refsum, H., Rosenberg, I.H., Ueland, P.M.) 3–9 (Kluwer Academic Press, Boston, 1997).
6. Heinecke, J.W., Rosen, H., Suzuki, L.A. & Chait, A. The role of sulfur-containing amino acids in superoxide production and modification of low density lipoprotein by arterial smooth muscle cells. *J. Biol. Chem.* **262**, 10098–10103 (1987).
7. Hogg, N. The effect of cysteine on the auto-oxidation of homocysteine. *Free Radic. Biol. Med.* **27**, 28–33 (1999).
8. Han, Q. *et al.* Homogeneous enzymatic colorimetric assay for total cysteine. *Clin. Chem.* **50**, 1229–1231 (2004).
9. Tan, Y. *et al.* Automated enzymatic assay for homocysteine. *Clin. Chem.* **49**, 1029–1030 (2003).
10. Han, Q. *et al.* Homogeneous, nonradioactive, enzymatic assay for plasma pyridoxal 5′-phosphate. *Clin. Chem.* **48**, 1560–1564 (2002).
11. Tan, Y. *et al.* Total-homocysteine enzymatic assay. *Clin. Chem.* **46**, 1686–1688 (2000).
12. Refsum, H. *et al.* Facts and recommendations about total homocysteine determinations: an expert opinion. *Clin. Chem.* **50**, 3–32 (2004).
13. El-Khairy, L. *et al.* Plasma total cysteine as a risk factor for vascular disease: The European Concerted Action Project. *Circulation* **103**, 2544–2549 (2001).
14. Tanaka, H., Imahara, H., Esaki, N. & Soda, K. Determination of L-cysteine with L-methionine γ-lyase. *Agric. Biol. Chem.* **45**, 1021–1022 (1981).
15. Pfeiffer, C.M., Huff, D.L. & Gunter, E.W. Rapid and accurate HPLC assay for plasma total homocysteine and cysteine in a clinical laboratory setting. *Clin. Chem.* **45**, 290–292 (1999).
16. Pastore, A. *et al.* Fully automated assay for total homocysteine, cysteine cysteinylglycine, glutathione, cysteamine, and 2-mercaptopropionylglycine in plasma and urine. *Clin. Chem.* **44**, 825–832 (1998).
17. Tang, L. & Tan, Y. Synthesis of *N,N*-disubstituted-*P*-phenylenediamine. U.S. Patent 6,448,446. (2002).
18. Tan, Y. *et al.* Overexpression and large-scale production of recombinant L-methionine-α-deamino-γ-mercaptomethane-lyase for novel anticancer therapy. *Protein Expr. Purif.* **9**, 233–245 (1997).
19. Xu, M. & Han, Q.H. High expression and production of high specific activity recombinant *S*-adenosylhomocysteinase (SAHH) and improved assays for *S*-adenosylmethionine (SAM). U.S. Patent 6,713,273. (2004).
20. Linnet, K. Performance of Deming regression analysis in case of misspecified analytical error ratio in method comparison studies. *Clin. Chem.* **44**, 1024–1031 (1998).
21. Bland, J.M. & Altman, D.G. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* **1**, 307–310 (1986).
22. Dello Strologo, L., Laurenzi, C., Legato, A. & Pastore, A. Cystinuria in children and young adults: success of monitoring free-cystine urine levels. *Pediatr. Nephrol.* **22**, 1869–1873 (2007).