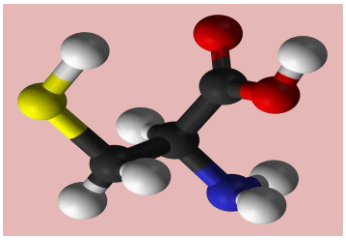
A/C DIAGNOSTICS ENZYMATIC L-CYSTEINE ASSAY



[--96 well-plate format--]

[The A/C Enzymatic L-Cysteine Assay is intended for the quantitative in vitro diagnostic determination of L-cysteine (CYS) in total cysteine in plasma or serum, tissues and cell extracts. The device will be monitored L-cysteine concentrations in plasma that could help to diagnosis, prevent and treatment of obesity and related with cardiovascular and other diseases.

The A/C Enzymatic Vitamin L-cysteine Assay is for research use only.

[CAT NO. AC-CYS-80]

80 sample Tests

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A/C ENZYMATIC CYSTEINE ASSAY

1. INTENDED USE

A/C Enzymatic Cysteine Assay is intended for the quantitative determination of total cysteine in plasma, tissues and cell extracts.

2. GENERAL DESCRIPTION

Elevated plasma total homocysteine (tHcy) is a risk factor for cardiovascular disease. Cysteine is structurally similar and metabolically linked to tHcy. Recent studies have demonstrated that plasma total cysteine (tCys) levels are a risk factor of vascular disease in the coronary, cerebral, and peripheral vessels (1). Cysteine (CYS) is another sulfhydrl-containing amino acid with structural and chemical properties similar to those of HCY. Autoxidation of CYS *in vitro* promotes several processes considered to be involved in atherogenesis and thrombogenesis. CYS has a cytotoxic effect *in vitro* against several cell types. CYS supports superoxide-mediated modification of LDL, which may facilitate foam cell formation. Finally, CYS forms an adduct with nitric oxide and may thereby impair endothelial function (2-4).

Recent studies have demonstrated that plasma total cysteine (tCys) levels are a risk factor of vascular disease in the coronary, cerebral, and peripheral vessels. People with high levels of the amino acid cysteine carry 6 -10 kilograms more fat than other people. Professor Refsum's research indicate that cysteine plays a key role in how the body metablises energy, stores fat, and breaks down fat (5-6).

So measurement of plasma total cysteine levels is very impotent to studying this phenomenon are generating knowledge which could help to prevent and treat life-threatening obesity.

3. PRINCIPLE OF ASSAY

In the A/C Enzymatic Cysteine Assay, two recombinant enzymes are used: S-adenosylhomocysteine hydrolase (rSAHH) cloned from *T. vaginalis* and L-methionine-α-deamino-γ-mercaptomethane-lyases (rMETase) cloned from *Pseudomonas putida*. The rMETase reacts with L-cysteine to form hydrogen sulfide. (7, 8). Since this enzyme also converts homocysteine to H_2S, so homocysteine is first removed by SAHH which combine HCY and adenosine to form s-adenosyl-L-homocysteine (ado-Hcy) to avoid interference the assay.

In the first step, samples are reduced by dithiothreitol (DTT) to generate free reduced Cys and Hcy. Simultaneous use of SAHH with excess adenosine converts the reduced Hcy to SAH.

Step 1

$$\text{Reduced Hcy + Ado} \xrightarrow{\text{rSAHH}} \text{Ado-Hcy} + H_20$$

Step 2.

$$\text{Reduced Cys} \xrightarrow{\text{rMETase}} \text{Pyruvate} + H_2S + NH_3$$

Step 3

H_2S Combines with DBPDA to form an absorbent compound.

The absorbance is read at 675 nm.

$$\text{DBPDA} + H_2S \xrightarrow[\text{HCl}]{K_3Fe(CN)_6} \text{3,7-Bis (dibutylamino) phenothiazine-5 chloride}$$

The chromophore DBPDA reacts specifically with hydrogen sulfide to give a fluorescence or absorbance reading. Absorbance can be read between 660 and 680 nm. For fluorescence the excitation wavelength is 665 nm and the emission is at 690 nm (9-11).

4. REAGENTS SUPPLIED AND PREPARATION

Reagent Kit, 80 Samples Tests in duplicate

Reagents	Quantity	Reconstitution
Assay Buffer	2 vials (40 ml/each) Storage at 2-8°C	See preparation of Working RI
R1	2 vials (Lyophilized powder) and storage at -20°C	See preparation of Working RI
R2	2 vials (Lyophilized powder) and storage at -20 °C	see preparation of working R2
Chromogen R3	1 vial, 7 ml.	Ready to use
Chromogen R4	1 vial, 5 ml	Ready to use
Controls Low/High levels	2 vials, 120 µl/vial	Ready to use
Calibrators	3 vials, 120 µl/vial	Ready to use

5. REAGENT STORAGE CONIDITION

1. All kit components that are stores at their recommended storage conditions are stable until the expiration date on their label. Do not use past their expiration.

2. The lyophilized powder RI and R2 should be stored at –20°C, and the enzymes are stable for 6 months.
3. All other reagents are stored at 2-8°C in their original containers.

6. SPECIMEN COLLECTION

1. Aseptically collect blood samples by venipuncture and draw blood into an recommended.
2. Plasma containing visible particulate matter should be spun down utilizing high-speed centrifugation before run assay.
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. Avoid using hemolyzed, lipemic, or bacterially contaminated plasma.

7. EQUIPMENTS REQUIRED, BUT NOT PROVIVIDED

1. 96-well microplates.
2. Multiple pipettes
3. Incubator/shaker for microplate
4. Absorbance reader with filter wavelength at 660-680 nm. (Recommend wavelength at 675nm).

8. PREPARATION OF REAGENTS

9.

1. Working R1: a vial of R1 is dissolved in 25 ml of Assay Buffer, then mix and stand on ice. Ready for a 96-well plate use. The solution is stable at -20°C for one week.
2. Working R2: a vial of R2 is dissolved in 7 ml of assay buffer.
3. R3 and R4 are ready for use.

10. TEST PROCEDURES

1. For each the calibrators and quality controls and samples should be run in duplicate. The addition of all reagents in the assay must be consistent. It is suggested that, pipetting should be in the same ordered from well to well, and at same rate. Check software and reader requirements for the correct Calibrators/Controls configurations.
2. Transfer 10 µl of samples including calibrators and QC's to the reaction wells. Use a multiple pipettes. Add 200 µl of working R1 to each well. Shaking and incubate at 37°C for 20 minutes.

3. Add 40 µl of working R2 to each well of the reaction plate. Shaking and incubate at 37°C for 10 minutes.

4. Add 25 µl of chromogen RII and 15 µl of chromogen RIII, shaking and incubate reaction plate at 37°C for 10 minutes.

5. Read the plate using a Reader with a filter wavelength at 660-680nm. The total cysteine values are calculated according to the calibration curve.

96-well plate layout

C1	C1	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
C2	C2	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
C3	C3	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
CL	CL	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
CH	CH	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40
S1	S1	S9	S9	S17	S17	S25	S25	S33	S33	S41	S41
S2	S2	S10	S10	S18	S18	S26	S26	S34	S34	S42	S42
S3	S3	S11	S11	S19	S19	S27	S27	S35	S35	S43	S43

CALCULATION OF RESULTS

If a 96-well plate absorbance reader with built in data calculation program is used, refer to the manual for the plate reader and create a linear regression program using the concentrations stated on the labels of each of the calibrators
For manual evaluation, a calculation curve is constructed linear regression plot by linear-linear graph paper, and plotting the average absorbance values obtained for each the Calibrator against the corresponding concentration (µmol/L). The unknown L-cysteine concentrations can then be read from the calibration curve using the average absorbance values of each sample.
For automatic or manual calculation of A/C Enzymatic Vitamin B6 Assay results refer to following procedures:

1. Calculate the mean absorbance value for each sample in duplicate including calibrators, controls and samples,

2. Construct a calibration curve by plotting the average OD obtained for each calibrator against its concentration of L-cysteine in µmol/L to linear regression.
3. Use the mean OD values for each specimen to determine the corresponding concentrations of cysteine on the horizontal or X axis.

11. PERFORMANCE CHARACTERISTICS

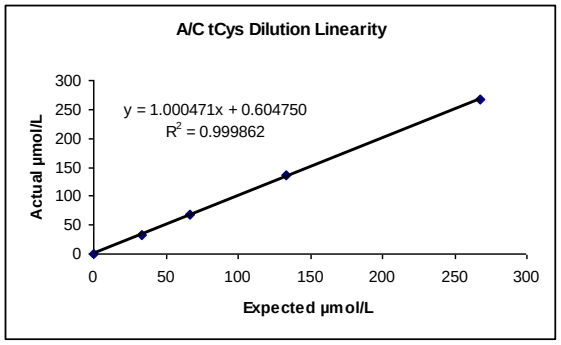
1. Sensitivity: the lower limit of detection (LLD) of A/C enzymatic tCYS assay is 16.9 µmol/L. The lower limit is defined as the concentration at three standard deviations from the mean of the blank solution.

2. Recovery: Recovery of various concentrations of L-cysteine spiked in human plasma is 97.37 $\pm$ 4.10 %.

3. Linearity and analytical range
Plasma sample can be diluted with blank solution at least up 1:8 to obtain linear result (Figure 1). The calibration curve is linear for at least up to 600 µmol/L.

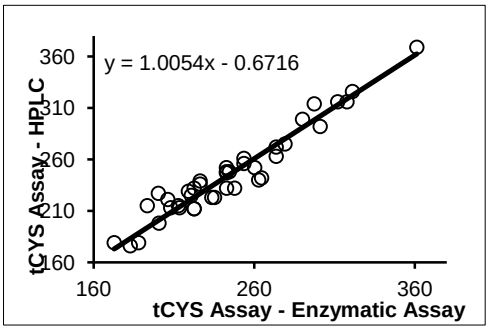
4. Precision: The mean with-in and between-run CVs were 4.4% and 4.8%, respectively. The measured values in plasma samples by the enzymatic CYS assay highly correlated with an HPLC method. Deming regression analyses for the enzymatic assay (x) vs. the HPLC method (y) were as follows: y=1.0054x – 0.6716; r^2= 1.0054 (n= 40) (Figure 2.).

Figure 1.



Linearity was assessed on the A/C tCys assay with microplate format by making serial dilutions of human plasma with 267.3 µmol/L.

Figure 2. The Correlation of Vitamin B6 between A/C tCys Assay and HPLC Method



11. PATENTS FOR THE A/C tCYS ASSAY

A/C Diagnostics has filed worldwide patent applications for the A/C Enzymatic Cysteine Assay.

12. REFERENCES

1. El-Khairy L, Ueland PM, Refsum H, Graham IM, Vollset SE. Plasma total cysteine as a risk factor for vascular disease: The European Concerted Action Project. Circulation **103**, 2544-2549, 2001.
2. Heinecke JW, Rosen H, Suzuki LA, et al. The role of sulfur-containing amino acids in superoxide production and modification of low-density lipoprotein by arterial smooth muscle cells. J Biol Chem. 1987; 262:10098-10103.
3. Hogg N. The effect of cysteine of cysteine on the auto-oxidation of homocysteine. Free Radic Biol Med 1999; 27:28-33.
4. Welch GN, Upchurch GR, Loscalzo J. Homocysteine, oxidative stress, and vascular disease. Hosp Pract. 1997; 32:81-92.
5. Elshorbagy, AK. Kozich, V., Smith, D., and Refsum, H., Cysteine and obesity. Obesity (2012) 20, 473-481.
6. Elshorbagy, AK. Kozich, V., Smith, D., and Refsum, H., Cysteine and obesity: consistency of the evidence across epidemiologic, animal and cellular studies. Clin Nutr. V.15. No.1 Jan. 2012. 49-57.
7. Tan Y, and Hoffman RM. Over-expression and large-scale production of recombinant L-methionine-α-deamino -γ-mercaptomethane-lyase for novel anticancer therapy. Protein Purification and Expression **9**, 233-245, 1997.
8. Han Q, and Hoffman RM. High expression, purification and properties of recombinant homocysteine, α, γ-lyase. Protein Expression and Purification **14**, 267-274, 1998.
9. Han, Q, et al. Homogeneous Enzymatic Colorimetric Assay for Total Cysteine. Clinical Chemistry 2004; v. 50, p.1229-1231.
10. Han, Q., and Hoffman, R.M. Enzymatic assay for total plasma Cys. Nature Protocols 3, 1778-1781, 2008.
11. Tan Y, and , Hoffman RM. Total-homocysteine enzymatic assay. Clin. Chem. **46**, 1686-1688, 2000.