



EAGLE
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Homocysteine HPLC Assay

Catalog Number: HCY31-H100

100 Tests

For Research Use Only. Not for use in diagnostic procedures.

v. 1.0

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1. Intended purpose

The Eagle Biosciences Homocysteine HPLC Assay Kit is intended for the quantitative determination of homocysteine in plasma and serum. The Homocysteine HPLC Assay Kit is for research use only and should not be used for diagnostic procedures.

2. Introduction

Homocysteine is an amino acid, which is produced out of methionine. It is not ingested by nutrition. Homocysteine is rapidly degraded to cysteine when vitamin B6, folic acid and vitamin B12 are sufficiently available. Increased levels of homocysteine will damage heart and blood vessels. Meanwhile it is an established risk marker for cardiovascular disease. US studies demonstrate that 40% of heart attacks are caused by increased homocysteine levels. Also the risk for stroke is increased by elevated homocysteine concentrations in plasma. A participation of homocysteine in Alzheimer and Parkinson diseases are under discussion.

The reason for high homocysteine levels are a lack of vitamin B6, folic acid and vitamin B12 and/or a defect in the enzyme cystathione synthase, which catalysis the reaction from homocysteine to cysteine. Increased homocysteine concentration have been found to be reduced by the supplementation with folic acid, vitamin B6 and vitamin B12.

The Eagle Biosciences Homocysteine HPLC Assay Kit makes it possible to determine homocysteine in an easy, fast and precise method. The Homocysteine HPLC Assay kit includes all reagents for preparation and separation of the samples with exception of the columns (IC2801rp) and the controls (IC2801ko). Both can be supplied by Eagle Biosciences. Beside the complete test kits it is possible to order all components separately. Please request our single component price list.

3. Warnings and precautions

- All reagents of this Homocysteine HPLC Assay Kit are strictly intended for research use only.
- Test kit and column are concerted. Using alternative columns might cause in insufficient separation, resulting in false high results. The given test characteristics might not be fulfilled.
- Do not interchange the Homocysteine HPLC Assay Kit components from different lots.
- Calibrator and controls contain human plasma. It was tested and found negative for HBsAg, anti-HIV-1/2, and anti-HCV. No test can guarantee the absence of HBsAg or HIV, and so all human serum based reagents in this kit must be handled as though capable of transmitting infection.
- The precipitating reagent contains acid and has to be handled carefully. It is corrosive and causes burns. It should be handled with gloves, eye protection, and



appropriate protective clothing in a hood. Any spill should be wiped out immediately with copious quantities of water. Do not breathe vapor and avoid inhalation. In case of an accident or indisposition contact immediately a physician.

- Wear disposable gloves while handling specimens or kit reagents and wash hands thoroughly afterwards.
- Do not pipette by mouth.
- Do not eat, drink, smoke or apply makeup in areas where specimens or kit reagents are handled.
- Reagents should not be used beyond the expiration date shown on kit label.
- Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera. During handling of all kit reagents, controls and serum samples observe the existing legal regulations.

4. Materials Provided

Article no.	Component	Designation	Amount
IC2801lm	ELU	Mobile phase	1000 ml
IC2801ka	CAL	Calibrator (lyoph. 1 ml)	1 vial
IC2801is	IS	Internal standard (lyoph. 12 ml)	1 vial
IC2801rl	RED	Reduction solution (lyoph.)	1 vial
IC2801fr	PREC	Precipitation reagent	12 ml
IC2801re	RECON	Reconstitution solution	25 ml
IC2801dl	DERIVAT	Derivatisation solution	12 ml

5. Additional Special Equipment

- 1.5 ml reaction tubes (Eppendorf)
- Centrifuge
- Various pipettes
- HPLC with Fluorescence-detector
- HPLC column Homocysteine (IC2801rp)
- Heatable shaker or water bath
- Vortex mixer



6. Reagent preparation

- Reconstitute the **calibrator (CAL)** in **1 ml** reconstitution solution (RECON), divide the calibrator in several portions and store them at -20 °C. Avoid repeated freeze-thaw circles. The concentration of homocysteine might have minor changes from lot to lot.
- Reconstitute the **internal standard (IS)** in **12 ml** reconstitution solution (RECON). Take aliquots and store them at -20 °C. Avoid repeated freeze-thaw circles.
- Reconstitute the **reduction solution (RED)** in the volume of reconstitution solution (RECON) given on the label and store at 2-8 °C. The reconstituted reduction solution is stable for 3 months.
- All other test reagents of the Homocysteine HPLC Assay Kit are stable at 2-8 °C, up to the date of expiry stated on the label.

7. Specimen

- EDTA-plasma drawn from venous fasting blood could be used in this test system. EDTA-plasma is preferred because in serum a clotting time dependent increase in homocysteine concentration is observed.
- The sample should be centrifuged (2000 g, 10 min., 2-8°C) within 30 min after venipuncture and stored directly at 2-8°C. For long time storage the samples should be frozen at -20°C.

8. Procedure

Principle of the method

For the determination of homocysteine, the sample is reduced and derivatized in one step. The albumin bound and the oxidized homocysteine is reduced and converted into a fluorescent probe. During a precipitation step high molecular substances are removed. The sample is cooled at 2-8°C, centrifuged and injected into the HPLC system. The isocratic separation via HPLC at 30°C uses a "reversed phase" column. One run lasts 5 minutes. The chromatograms are recorded by a fluorescence detector. The quantification is performed by the delivered plasma calibrator; the concentration is calculated via the "internal standard method" by integration of the peak areas resp. peak heights.

Sample preparation

1. Pipette into 1.5 ml reaction tubes:



50 µl sample, CAL or CTRL

+

50 µl IS

+

20 µl RED

+

100 µl DERIVAT

2. Vortex briefly. Incubate for **10 min** at 60 °C
3. Cool down the sample at 2-8 °C and add 100 µl PREC
4. Vortex briefly, incubate for 5 min at 2-8°C and centrifuge at 10.000g for 5 minutes.
5. Inject 20 µl of the supernatant into the HPLC-system. The supernatant is stable in the dark for 6 days at 2-8°C.

Chromatographic settings

Column material:	MZ Inertsil ODS-2, 5 µm
Column dimension:	125 mm x 4 mm
Flow rate:	0.7-1.0 ml/min
Fluorescence detection:	Excitation 385 nm
	Emission 515 nm
Injection volume:	20 µl
Running time:	5 min
Temperature:	30 °C

Treatment of the HPLC column

After the analysis the column should be flushed with 15 ml deionized water (1 ml/min) and stored in 50% methanol in deionized water (ca. 15 ml, flow 0.7 ml/min). Before use, the system should be equilibrated with ca. 30 ml ELU.

9. Calculation of analytical results

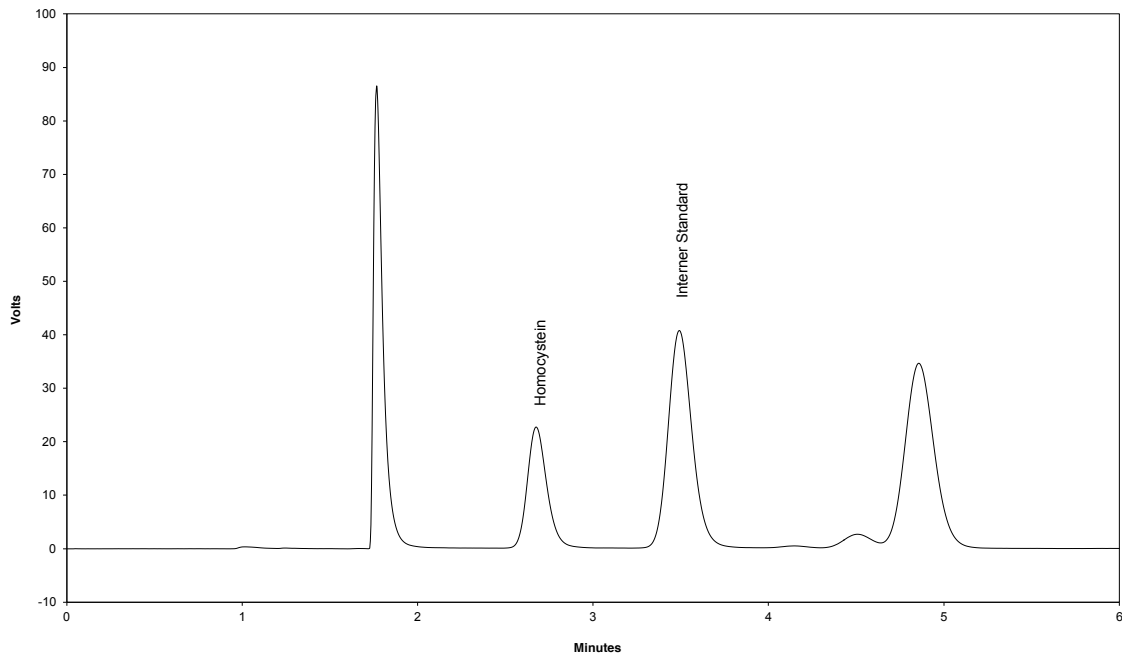
Calculation



$$\text{Conc. sample } (\mu\text{mol/l}) = \frac{\text{peak area patient} * \text{conc. calibrator } (\mu\text{mol/l})}{\text{peak area IS patient}} * F$$

$$F = \frac{\text{Peak area IS of the calibrator}}{\text{Peak area homocysteine of the calibrator}}$$

Typical chromatogram



10. Internal quality control

Reference intervals

Homocysteine values: (Resch "Homocysteine" ISBN 3-920328-17-5)
< 15 $\mu\text{mol/l}$ Normal value

We recommend that each laboratory should develop their own normal range. The values mentioned above are only for orientation and can deviate from other published data.



11. Validation data

Precision and reproducibility

Intra-Assay CV:	0.9 % (8.7 $\mu\text{mol/l}$)	[n = 6]
	1.0 % (23.4 $\mu\text{mol/l}$)	[n = 6]
Inter-Assay CV:	3.5 % (8.9 $\mu\text{mol/l}$)	[n = 6]
	1.7 % (23.2 $\mu\text{mol/l}$)	[n = 6]

Linearity

up to 1000 $\mu\text{mol/l}$

Detection limit

0.3 $\mu\text{mol/l}$

Recovery

97.3 %

12. Limitations of the method

Since the concentration of homocysteine in serum samples is depending on the clotting time, EDTA-plasma should be preferred as sample.

13. Disposal

The mobile phase (ELU), reduction solution (RED), internal standard (IS), and derivatisation solution (DERIVAT) must be disposed as non-halogenated solvent. The precipitation reagent (PREC) could be neutralized with NaOH and if the pH value is neutral it can be disposed as salt solution. Please refer to the appropriate national guidelines.



14. Troubleshooting

Problem	Possible reason	Solution
No signal	No or defect connection to evaluation system	Check signal cord and connection
	Detector lamp is altered	Change lamp
No peaks	Injector is congested	Check Injector
Double peaks	Dead volume in fittings and / or column	Renew fittings and / or column
Contaminating peaks	Injector dirty	Clean injector
	Contamination at the head of the column	Change direction of the column and rinse for 30 min at low flow rate (0.2 ml/min) with mobile phase
	Air in the system	Degas pump
	Autosampler vials contaminated	Use new vials or clean them with methanol
Broad peaks, tailing	Precolumn / column exhausted	Use new precolumn / column
Variable retention times	Drift in temperature	Use a column oven
	Pump delivers imprecise	Check pump, degas the system
	System is not in steady state yet	Rinse system mobile phase for 15 min
Baseline is drifting	Detector lamp did not reach working temperature yet	Wait
	Detector lamp is too old	Renew lamp
Continue baseline is drifting	System is not in steady state yet	Rinse system mobile phase for 15 min
	Pump delivers imprecise	Check pump, degas the system
Baseline is not smooth	Pump delivers imprecise	Check pump, degas the system
	Detector flowcell is dirty	Clean flow cell



15. Literature references

- Role of Plasma Homocysteine in Arterial Occlusive Diseases. (1994) Clinical Chemistry, Vol. 40 No. 6, pp. 857-858.
- Association between Plasma homocysteine concentrations and extracranial carotid-artery stenosis. Selhub, J. et al. (1995). The New England Journal of Medicine, Feb. 95, Vol. 332 No. 5, pp. 286-291.
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- Hormone replacement therapy may reduce high serum homocysteine in postmenopausal women. van der Mooren, M. (1994). European Journal of Clinical Investigation, Vol. 24, pp. 733-736.
- Homocysteine metabolism in pregnancies complicated by neural-tube defects. Mills, J. et al. (1995). The Lancet, Jan. 1995, Vol. 345, pp. 149-151.

For further information about this kit, its application or the procedures in this insert, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.