



Glutathione HPLC Assay

Catalog Number: GLU31-H100

100 Tests

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

v. 1.0

EAGLE BIOSCIENCES, INC.

20A Northwest Blvd., Suite 112, Nashua, NH 03063

Phone: 866-419-2019 Fax: 617-419-1110

www.EagleBio.com



1. Intended purpose

The Eagle Biosciences Glutathione HPLC Assay Kit is intended for the quantitative determination of glutathione in EDTA blood. The Glutathione HPLC Assay Kit is for research use only and should not be used for diagnostic procedures.

2. Introduction

Glutathione (GSH) an intracellular tripeptide consisting of glycine, cysteine and glutamic acid. It is common in all tissues and protects the cells against oxidative stress. It is important for the activation or inhibition of enzymes and transport proteins. The transport of amino acids is also controlled by glutathione. Glutathione is very important for the stabilization of protein and non-protein sulfhydryl-groups to maintain a reducing intracellular environment.

Most of the intracellular glutathione is reduced (approx. 90 % in EDTA-blood), only 10 % is oxidized (GSSG). The NADPH-dependent glutathione reductase maintains this steady state. Alterations in the glutathione status are involved in the pathogenesis of several diseases. In discussion are reperfusion damage, liver injury, cancer, diabetes mellitus, cataract, inflammatory diseases, chronic lymphatic edema and radiation damages. Altered glutathione concentrations might also be due to pollution, cigarette smoke, side effects of drugs and aging. In case of oxidative stress the amount of reduced glutathione is diminished. The relation of reduced to oxidized glutathione gives information about the redox and detoxification status of cells and tissue.

The Eagle Biosciences Glutathione HPLC Assay Kit makes it possible to determine glutathione in an easy, fast and precise way. The Glutathione HPLC Assay Kit includes all reagents in ready to use form for preparation and separation of the samples with exception of the columns (IC1800rp) and the controls (IC1800ko). Both can be supplied by Eagle Biosciences. Beside the complete test kit, it is possible to order all components separately. Please request our single component price list.

3. Warnings and precautions

- All reagents of this Glutathione 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 Glutathione 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 of the Glutathione HPLC Assay Kit 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
IC1800lm	ELU	Mobile phase	1000 ml
IC1800ka	CAL	Calibrator, (lyoph. 0.25 ml)	8 vials
IC1800is	IS	Internal standard	6 ml
IC1800rk	RECON	Reconstitution solution	5 ml
IC1800rb	REAC	Reaction buffer	27 ml
IC1800vl	SOL	Dilution solution	25 ml
IC1800rl	RED	Reduction solution (lyoph. 1.2 ml)	1 vial
IC1800dl	DERIVAT	Derivatisation solution	12 ml
IC1800fr	PREC	Precipitation solution	12 ml



5. Additional special equipment

- 1.5 ml reaction tubes (Eppendorf)
- Centrifuge
- Various pipettes
- HPLC with Fluorescence-detector
- HPLC column Glutathione (IC1800rp)
- Heatable shaker or water bath
- Vortex mixer

6. Reagent preparation

- Reconstitute the calibrator (CAL) in 0.25 ml reconstitution solution (RECON). The calibrator is for single use only; discard the rest of the material. The concentration of glutathione might have minor changes from lot to lot.
- Reconstitute the reduction solution (RED) in 1.2 ml reconstitution solution. The solution is then stable for 3 months at 2-8 °C.
- All other test reagents of the Glutathione HPLC Assay Kit are stable at 2-8 °C, up to the date of expiry stated on the label.

7. Specimen

- EDTA- blood could be used in this Glutathione HPLC Assay Kit.
- Glutathione is rather sensitive against oxidation. Sample transport should be at 2-8 °C.
- Samples are stable for at least 2 days at 2-8 °C or 2 weeks at -20 °C. With longer storage the content of oxidized glutathione increases. After thawing the samples have to be processed immediately.
- Please note: Before the assay, the samples have to be frozen to hemolyze the erythrocytes in order to release the glutathione from the cells.



8. Procedure

Principle of the method

For the determination of glutathione the sample is divided in two aliquots. One is reduced and the total amount of glutathione is measured. The other aliquot is treated without reduction solution, which determines only the reduced glutathione. During the derivatisation reaction glutathione is converted into a fluorescent probe. The following precipitation step removes high molecular substances. After centrifugation the fluorescent probe is cooled (2-8°C) and injected into the HPLC system. The isocratic separation via HPLC at 30°C uses a reversed phase column in two runs. One run lasts 4 minutes. The chromatograms are recorded by a fluorescence detector. The quantification is performed with the delivered EDTA-blood calibrator; the concentration is calculated by the internal standard method. The amount of oxidized glutathione is calculated by subtraction of:

$$\text{Glutathione}_{\text{total}} - \text{Glutathione}_{\text{reduced}}$$

Please take in mind that the difference must be divided by two because oxidized glutathione (GSSG) consists of two reduced GSH molecules.

Sample preparation

1. Pipette into 1.5 ml reaction tubes: **100 µl** sample, CAL or CTRL + **200 µl** SOL
2. Vortex briefly. The sample is then divided in two portions:

Total glutathione	Reduced glutathione
Add 50 µl diluted sample + 100 µl IS + 20 µl RED + 100 µl DERIVAT	Add 50 µl diluted sample + 100 µl REAC + 100 µl DERIVAT

3. Incubate for **20 minutes** at 60 °C.
4. Add **100 µl** PREC
5. Incubate for **10 minutes** at 2 -8 °C and centrifuge for **10 min** at 10.000 g.
6. Add **100 µl** supernatant to **200 µl** REAC in autosampler-vials
7. Inject **20 µl** in the HPLC-system.



Chromatographic settings

Column material:	MZ Inertsil ODS, 5 µm
Column dimension:	125 mm x 4 mm
Flow rate:	0.75-1.0 ml/min
Fluorescence detection:	Excitation 385 nm Emission 515 nm
Injection volume:	20 µl
Running time:	4 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 Eluent.

9. Calculation of analytical results

Calculation

Glutathione total

$$\text{Conc. sample} = \frac{\text{peak area patient} * \text{conc. calibrator}}{\text{peak area IS patient}} * F$$

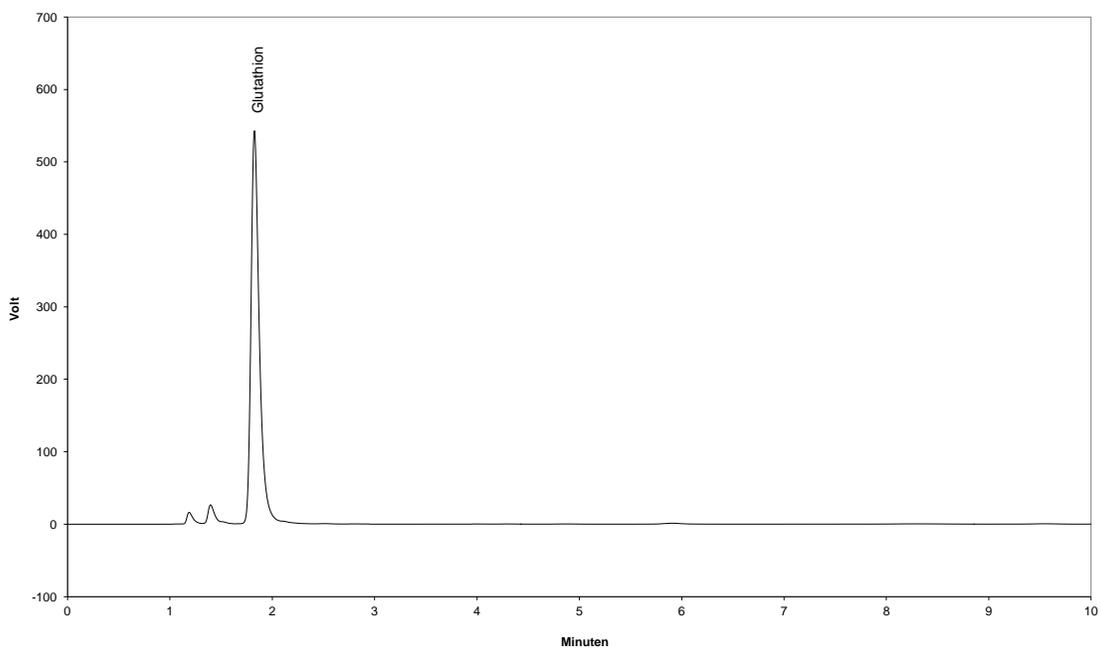
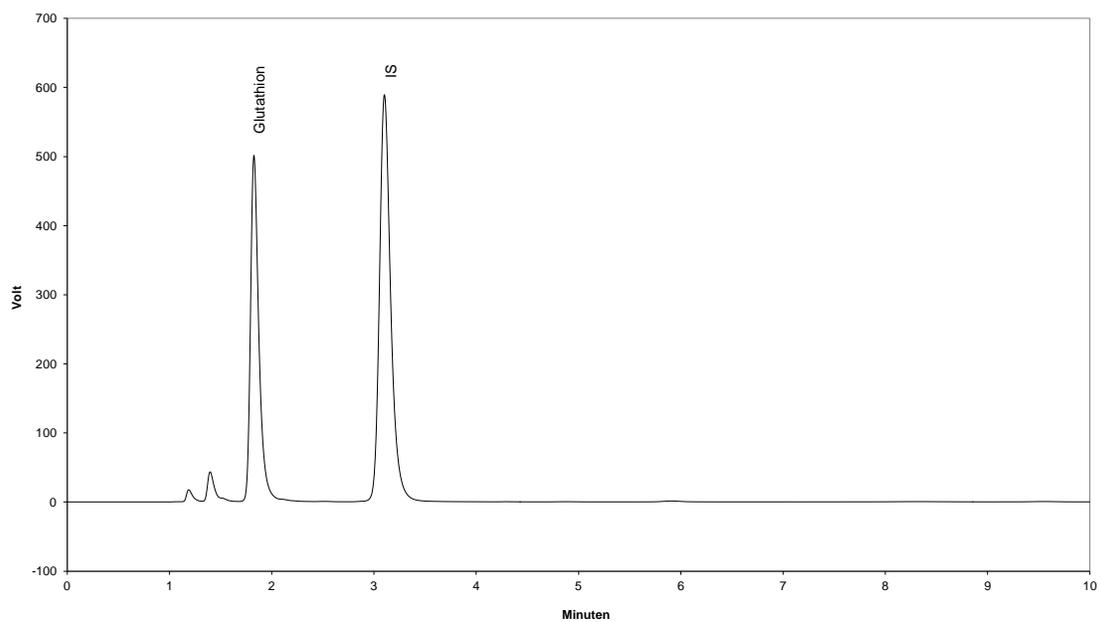
$$F = \frac{\text{peak area IS of the calibrator}}{\text{peak area of the calibrator}}$$

Glutathione reduced

$$\text{Conc. sample} = \frac{\text{peak area patient} * \text{conc. calibrator}}{\text{peak area calibrator}}$$



Typical chromatogram



10. Internal quality control

Reference intervals

GSH _{total} :	783 – 1346 $\mu\text{mol/l}$
GSH _{reduced} :	639 – 1146 $\mu\text{mol/l}$
GSH _{reduced/total} :	> 80 %



We recommend that each laboratory 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:	GSH _{total} :	1.2 % (551 µmol/l)	[n = 6]
	GSH _{reduced} :	1.2 % (286 µmol/l)	[n = 6]
Inter-Assay CV:	GSH _{total} :	2.8 % (554 µmol/l)	[n = 6]
	GSH _{reduced} :	3.5 % (271 µmol/l)	[n = 6]

Linearity

GSH _{total} :	up to 15 mmol/l
GSH _{reduced} :	up to 15 mmol/l

Detection limit

GSH _{total} :	2 µmol/l
GSH _{reduced} :	0.4 µmol/l

12. Limitations of the method

Serum or plasma should not be used because the content of glutathione is much low. It is not possible to distinguish between oxidized and reduced glutathione. Don't use lipemic samples.

13. Disposal

The mobile phase (ELU), reduction solution (RED), reaction buffer (REAC), internal standard (IS), and derivatisation solution (DERIVAT) must be disposed as non-halogenated solvent. The precipitation solution (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

- Siems et al. PZ Nr 45 141 Jahrgang 7 November 1996
- Piccoli et al. Journal of Chromatography A, 676 (1994) 239-246



- Henning S.M., J.Z. Zhang, R.W. McKee, M.E. Swendseid, R.A. Jacob. Glutathion blood levels and other oxidant defense indices in men fed diets low in vitamin C. American institute of nutrition, 1991, 1969-1975.
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- Meister A. Mitochondrial changes associated with glutathione deficiency. Biochim Biophys Acta 1995; 35-42.

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.