



Glutathione (GSH/GSSG) HPLC

For the determination of Glutathione in EDTA-blood

For Research Use Only. Not For Use In Diagnostic Procedures

Critical Changes: Please read carefully. e.g. Sample Preparation for now specifies
"mix well" during three steps.

Catalog Number:	30-1800
Size:	100 determinations
Version:	30.06.2008– ALPCO 8/26/08

ALPCO Diagnostics

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use the protocol included with the kit ONLY.**

1. INTENDED USE

This Assay is intended for the quantitative determination of glutathione in EDTA-blood. This assay is designed for research use only.

2. SUMMARY AND EXPLANATION OF THE TEST

Glutathione (GSH) is an intracellular tripeptide common in all tissues, which protects the cells against oxidative processes. It has important functions in several metabolic pathways like activation or inhibition of enzymes, and transport of molecules and at the transport of amino acids.

A very important function is stabilizing SH-groups in proteins and other molecules to maintain a reducing intracellular environment.

Alterations in the glutathione status are involved in the pathogenesis of several diseases including:

Reperfusion damage, liver injury, cancer, diabetes mellitus, cataract, inflammatory diseases, chronic lymphatic edema, radiation damages.

Altered glutathione concentrations might also be due to pollution, cigarette smoke, as well as side effects of drugs and aging.

Most of the cellular glutathione is reduced (in EDTA-blood approx. 90 %), only a minor amount (10 %) is oxidized (GSSG). This steady state is maintained by the NADPH-dependent glutathione reductase.

In case of oxidative stress, GSH is needed for several reactions of the primary and secondary anti oxidative protection.

3. PRINCIPLE OF THE TEST

The determination of glutathione starts by adding a dilution solution to the sample and dividing it into two aliquots.

The measurement of the reduced fraction is performed by the addition of reaction buffer and derivitization solution. After an incubation of 20 minutes, in which GSH is transformed into a fluorescent product, a precipitation solution is added to separate higher molecular substances.

The measurement of the total glutathione is performed by the addition of the reduction solution, internal standard and derivitization solution. After that, the sample is handled like the reduced fraction. Depending on the reduction solution, all the glutathione (GSH and GSSG) is reduced to GSH.

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20 µl of the supernatant are injected into the HPLC system.

The separation via HPLC follows an isocratic method at 30°C using a reversed phase column in two runs; one run lasts 10 minutes. The chromatograms are recorded by a fluorescence detector. The quantification is performed with the delivered EDTA-blood calibrator; the concentration is calculated via integration of the peak height by the external standard method for the reduced fraction and the internal standard method for the total GSH fraction. For the measurement of the reduced fraction, it is not possible to use the internal standard because of the production of mixed sulfides.

The amount of oxidized glutathione can be calculated by subtraction of:

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

Please remember that the difference must be divided by two because oxidized glutathione (GSSG) is built out of two GSH molecules.

Summary:

Besides many other parameters, the advantage of HPLC method lies in the simultaneous handling of many analytes in a single test. The HPLC system enables laboratories with or without experience in high performance liquid chromatography to use this technique for routine determination in a quick and precise manner. Unlike immuno assays with up to six calibrators per test, a one-point calibration is sufficient to calibrate the test system. It is also possible to automate the sample application and calculation of the results so that even higher numbers of samples can be handled quickly and easily.

4. MATERIAL SUPPLIED

Catalogue No	Kit Components	Quantity
KC 1800LM	Mobile phase, MOPHA	1000 ml
KC 1800KA	Calibrator, lyophilized, CAL	8 vials
KC 1800IS	Internal standard, INT STD	6 ml
KC 1800T	Reconstitution solution, RECSOL	5 ml
KC 1800VL	Dilution solution, DIL	25 ml
KC 1800RL	Reduction solution (lyoph. 1.2 ml), REDSOL	1 vial
KC 1800RB	Reaction buffer, REABUF	27 ml
KC 1800DL	Derivatization solution, DER	12 ml
KC 1800FR	Precipitation solution, PREC	12 ml
KC 1800KO	Control 1 + 2, 250 µl lyophilized, CTRL 1 + 2	2 x 3 vials

5. MATERIAL REQUIRED BUT NOT SUPPLIED

- 1.5 ml reaction tubes (Eppendorf)
- Centrifuge
- Various pipettes
- HPLC with Fluorescence-detector
- Reversed phase C₁₈-column
- Oven or water bath for heating at 60 °C

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6. PREPARATION AND STORAGE OF REAGENTS

- Reconstitute the **calibrator** in **250 µl** reconstitution solution. One vial is for **single use only**; discard the material which have not been used. The concentration of glutathione might have minor changes from lot to lot.
- Reconstitute the **controls** in **250 µl** reconstitution solution. Take the exact concentration from the product data sheet.
- The reduction solution should be resuspendend in 1.2 ml reconstitution solution. The solution is then stable for 3 months at 2-8 °C.
- The lyophilized reagents should be stored at -20 °C. All other test reagents are stable at 2-8 °C, up to the date of expiration stated on the label.

7. PRECAUTIONS

- For research use only.
- This product contain human source material which was tested and found to be non-reactive to HBsAg, anti-HIV-1/2, and anti-HCV. Since no method can offer complete assurance that hepatitis B virus, HIV-1/2, HVC or other infectious agents are absent, these reagents should be handled as if potentially infectious.
- The precipitation solution contains acid. Although diluted, it still must be handled with care. It can cause burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spill should be wiped out immediately with copious quantities of water. Do not breathe vapor and avoid inhalation.
- Reagents should not be used beyond the expiration date shown on kit label.

8. SPECIMEN COLLECTION AND PREPARATION

EDTA-blood is suited for this test system.

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 for 2 weeks at -20 °C. With longer storage, the content of oxidized glutathione increases.

Please note: Before analysis, lyse the erythrocytes by freezing and thawing in order to release the glutathione.

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9. ASSAY PROCEDURE

Procedural notes

- The quality control guidelines should be observed.
- Incubation time, incubation temperature and pipetting volumes of the different components are defined by the producer. Any variations of the test procedure, that are not coordinated with the producer, may influence the test results.
- The assay should always be performed according to the manual which is provided with the kit.

Sample and standard preparation

Pipette in a 1.5 ml reaction tube (e.g. Eppendorf):

100 µl sample, calibrator (CAL) or control (CTRL1, CTRL2) +

200 µl dilution solution (DIL)

Mix

Total-glutathione

50 µl diluted sample

+

100 µl internal standard (INT STD)

+

20 µl reduction solution (REDSOL)

+

100 µl derivitization solution (DER)

Reduced glutathione

50 µl diluted sample

+

100 µl reactions buffer (REABUF)

+

100 µl derivitization solution (DER)

Mix well, incubate for **20 minutes** at 60 °C.

Add **100 µl** precipitation solution (PREC), mix well

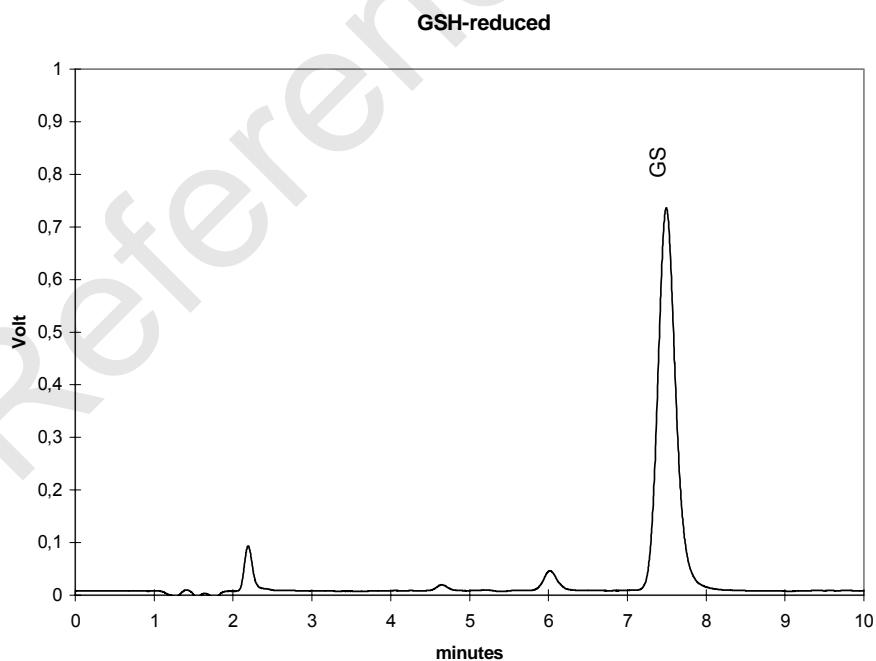
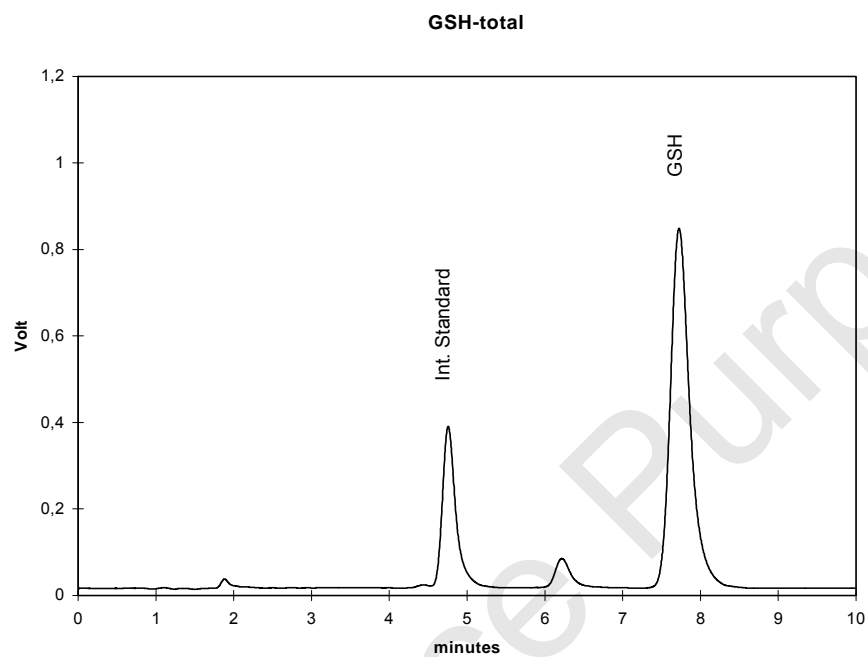
Precipitate for **10 minutes** at 2 -8 °C and centrifuge for **10 min** at 10.000 g

Add **200 µl** supernatant to **200 µl** reaction buffer (REABUF) in auto sampler-vials, mix well

Inject **20 µl** in the HPLC-system.

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Typical chromatogram



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12. LIMITATIONS

Do not use serum or plasma; the concentration of glutathione is very low. It is not possible to distinguish between oxidized and reduced glutathione in serum and plasma. Do not use lipemic samples.

13. QUALITY CONTROL

Expected values

(n = 50)

GSH (total)	763 – 1191 $\mu\text{mol/l}$
GSH (reduced)	620 – 970 $\mu\text{mol/l}$
GSH (reduced/total)	81 – 93 %

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

Controls

Control samples or serum pools should be analyzed with each run of calibrators and samples. Results generated from the analysis of the control samples should be evaluated for acceptability using appropriate statistical methods. In assays in which one or more of the quality control sample values lie outside the acceptable limits, the results for the sample may not be valid.

14. PERFORMANCE CHARACTERISTICS

Precision and reproducibility

Intra-Assay CV:	GSH _(total)	3.9 %	[n = 12]
	GSH _(reduced)	3.3 %	[n = 12]

Inter-Assay CV:	GSH _(total)	4.2 %	[n = 12]
	GSH _(reduced)	3.3 %	[n = 12]

Linearity

up to 10 mmol/l GSH_(total) and GSH_(reduced)

Detection limit

6 µmol/l GSH_(total) and GSH_(reduced)

15. DISPOSAL

The mobile phase (MOPHA), reduction solution (REDSOL), internal standard (INT STD), and derivitization solution (DER) must be disposed as non-halogenated solvents. The precipitation solution (PREC) can be neutralized with NaOH to pH 7.0 and disposed as a salt solution.
(Important: Reaction will produce heat, be careful)

Please refer to the appropriate national guidelines.

16. 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
	Auto sampler 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 imprecisely	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
	System is not in steady state yet	Rinse system mobile phase for 15 min
	Pump delivers imprecisely	Check pump, degas the system
Baseline is not smooth	Pump delivers imprecisely	Check pump, degas the system
	Detector flow cell is dirty	Clean flow cell

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17. REFERENCES

Henning S.M., J.Z. Zhang, R.W. McKee, M.E. Swendseid, R.A. Jacob. Glutathion blood levels and other oxidant defence indices in men fed diets low in vitamin C. American institute of nutrition, 1991, 1969-1975.

Rajender K.C., F.W. Lewis, M.H. Kutner, D.M. Bate, R.G.B. Roy, D. Rudman. Plasma cysteine, cystin, and glutathione in cirrhosis. Gastroenterology 1984; 87; 770-776

Meister A. Mitochondrial changes associated with glutathione deficiency. Biochim Biophys Acta 1995; 35-42.

18. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- The test components contain organic solvents. Contact with skin or mucous membranes has to be avoided.
- All reagents in the test package are to be used for research use only.
- The reagents should not be used after the date of expiry stated on the label.
- Single components with different lot numbers should not be mixed or exchanged.