Manual

Glutathione HPLC Kit

For the determination of glutathione in EDTA whole blood

Valid from 2018-06-07

REF **KCR1800**

Σ 100
(50 Bestimmungen + 50 Bestimmungen reduziertes Glutathion)

+8 °C
+2 °C

-20 °C
CAL
CTRL 1
CTRL 2
REDSOL

RUO

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Table of Contents

1. INTENDED USE

This HPLC application is intended for the quantitative determination of glutathione in EDTA whole blood. For research use only. Not for use in diagnostic procedures.

2. SUMMARY AND EXPLANATION OF THE TEST

Glutathione 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.

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

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 in two aliquots.

The measurement of the reduced fraction is performed by the addition of reaction buffer and derivatisation solution. After an incubation of 20 minutes, in which GSH is transformed into a fluorescent product, a precipitation reagent 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 derivatisation solution. After that, the sample is handled like the reduced fraction. The reduction solution reduces all the oxidised glutathione (GSSG) to each two molecules GSH, resulting in the measurement of **total GSH**.

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 area/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 oxidised glutathione (GSSG) can be calculated by subtraction of the reduced glutathione (GSH) from the total GSH. Please note that the obtained difference must be divided by two as oxidised glutathione (GSSG) is composed of two GSH molecules. The formula to be used is as follows:

$$\frac{\text{total GSH} - \text{GSH}}{2} = \text{GSSG}$$

Summary

This HPLC application allows the quantitation of total and reduced glutathione in an easy, fast, and precise way. The kit includes all reagents for a total of 100 determinations, i.e. 50 total GSH and 50 reduced glutathione (GSH). For the determination of total and reduced glutathione in a total of 100 samples 2 kits are required.

As for many other parameters, the advantage of HPLC analytics is the simultaneous handling of many analytes in a single test. The HPLC complete system enables even laboratories without experience in high performance liquid chromatography to use this technique for clinical chemical routines quickly and precisely. Mostly, a one-point calibration is sufficient for calibrating the test system – unlike immunoassays with up to 6 calibrators per test. It is possible to automate the sample application and calculation of the results so that even higher number of samples can be handled nearly without control. With short test series, the one-point calibration is much more economic than 6-point calibration for immunoassays.

4. MATERIAL SUPPLIED

Cat. No.	Label	Kit components	Quantity
KCR1800LM	MOPHA	Mobile phase	1000 ml
KCR1800KA	CAL	Calibrator (250 µl lyoph.; see specification data sheet for concentration)	8 vials
KCR1800IS	INT STD	Internal standard	6 ml
KCR1800RE	RECSOL	Reconstitution solution	15 ml
KCR1800VL	DIL	Dilution solution	25 ml
KCR1800RL	REDSOL	Reduction solution (1.2 ml lyoph.)	1 vial
KCR1800RB	REABUF	Reaction buffer	27 ml
KCR1800DL	DER	Derivatisation solution	12 ml
KCR1800FR	PREC	Precipitation solution (acid)	12 ml

Cat. No.	Label	Kit components	Quantity
KCR1800KO	CTRL 1 CTRL 2	Control 1 and 2 (250 µl lyoph.; see specification data sheet for concentration)	2 x 3 vials

The HPLC column (KCR1800RP) as well as individual components can be ordered separately from Immundiagnostik AG. Please ask for the price list of the individual components.

5. MATERIAL REQUIRED BUT NOT SUPPLIED

- Ultra pure water*
- 1.5 ml reaction tubes (e. g. Eppendorf)
- Centrifuge
- Various pipettes
- HPLC with fluorescence detector
- Reversed phase C_{18} column
- Water bath

* Immundiagnostik AG recommends the use of Ultra Pure Water (Water Type 1; ISO 3696), which is free of undissolved and colloidal ions and organic molecules (free of particles > 0.2 µm) with an electrical conductivity of 0.055 µS/cm at 25 °C (≥ 18.2 MΩ cm).

6. STORAGE AND PREPARATION OF REAGENTS

- **The lyophilised calibrator** (CAL) is stable at **-20 °C** until the expiry date stated on the label. Before use, the CAL has to be reconstituted with **0.25 ml reconstitution solution** (RECSOL). The concentration of glutathione slightly changes from lot to lot, the exact concentration is stated on the specification data sheet. **Calibrator** (reconstituted CAL) **is not stable and cannot be stored.**
- **The lyophilised controls 1 and 2** (CTRL 1 and CTRL 2) are stable at **-20 °C** until the expiry date stated on the label. Before use, they have to be reconstituted with each **0.25 ml reconstitution solution** (RECSOL). The concentration of glutathione slightly changes from lot to lot, the exact concentration is stated on the specification data sheet. **Controls** (reconstituted CTRL 1 and 2) **are not stable and cannot be stored.**
- **The lyophilised reduction solution** (REDSOL) is stable at **-20 °C** until the expiry date stated on the label. Before use, the REDSOL has to be reconstituted with **1.2 ml reconstitution solution** (RECSOL). **Reduction solution** (reconstituted REDSOL) can be stored for **3 months at 2–8 °C**.

- All other test reagents are ready to use. Test reagents are stable until the expiry date (see label of test package) when stored at **2–8 °C**.

7. PRECAUTIONS

- Human materials used in kit components were tested and found to be negative for HIV, Hepatitis B and Hepatitis C. However, for safety reasons, all kit components should be treated as potentially infectious.
- The precipitation reagent consists of an acid. It can cause burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spill should be wiped up immediately with copious quantities of water. Do not breath vapour and avoid inhalation.
- Reagents should not be used beyond the expiration date stated on kit label.

8. SPECIMEN COLLECTION AND PREPARATION

EDTA-blood is suited for this test system.

Glutathione is quite sensitive against oxidation. Samples should be transported at 2–8 °C.

Samples are stable for at least 2 days at 2–8 °C or for 2 weeks at -20 °C. During longer storage, the content of oxidised glutathione increases.

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

9. ASSAY PROCEDURE

Procedural notes

- Control samples should be analyzed with each run.
- Incubation time, incubation temperature and pipetting volumes of the components are defined by the producer. Any variation of the test procedure, which is not coordinated with the producer, may influence the results of the test. Immundiagnostik AG can therefore not be held responsible for any damage resulting from incorrect use.
- The assay should always be performed according the enclosed manual.

Test procedure

Sample dilution

Pipet **100 µl sample, calibrator** or **control 1 or 2** in 1.5 ml reaction tubes (e. g. Eppendorf)
Add **200 µl dilution solution** (DIL) and mix

Sample processing

Total GSH	Reduced glutathione
50 µl of the diluted sample	**50 µl** of the diluted sample
100 µl internal standard (INT STD)	**100 µl** reaction buffer (REABUF)
20 µl reduction solution	**100 µl** derivatisation solution (DER)
100 µl derivatisation solution (DER)	
Mix well, incubate for **20 min** at **60 °C**	
Add **100 µl** precipitation reagent (PREC) , mix well	
Precipitate for **10 min** at **2–8 °C** , then centrifuge for **10 min** at **10 000 *g***	
Pipet **200 µl** reaction buffer (REABUF) into an autosampler vial and add **200 µl** supernatant, mix well	
Inject **20 µl** into the HPLC system	

Chromatographic conditions

Column material:	MZ Inertsil ODS-2; 5 µm	
	MZ PerfectBond ODS-2; 5 µm	
	Bischoff Prontosil Eurobond; 5 µm	
Column dimension:	125 × 4 mm	
Flow rate:	0.75–1.0 ml/min	
Fluorescence detection::	Excitation:	385 nm
	Emission:	515 nm
Temperature:	30 °C	
Injection volume:	20 µl	
Running time:	~ 10 min	

It is recommended to use a guard column to extend column life.

10. TREATMENT OF THE COLUMN

After analysis, the column should be flushed with 30 ml ultra pure water (1 ml/min) and stored in 50% methanol in water (~ 30 ml, flow 0.7 ml/min). Before use, the system should be equilibrated with ~ 30 ml mobile phase (MOPHA).

11. RESULTS

Calculation

Total GSH

$$\frac{\text{Peak height sample} \times \text{calibrator concentration*}}{\text{Peak height internal standardy of the sample}} \times F = \text{sample concentration}$$

$$F = \frac{\text{Peak height interal standard of the calibrator}}{\text{Peak height calibrator}}$$

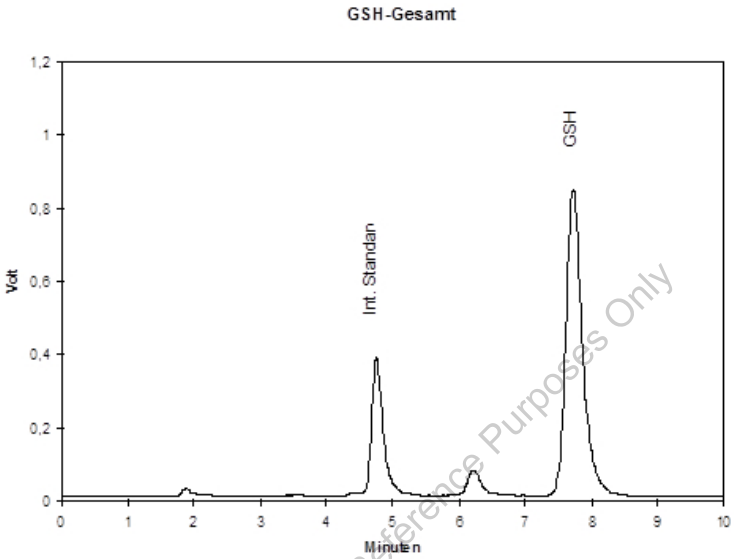
Reduced glutathione (GSH)

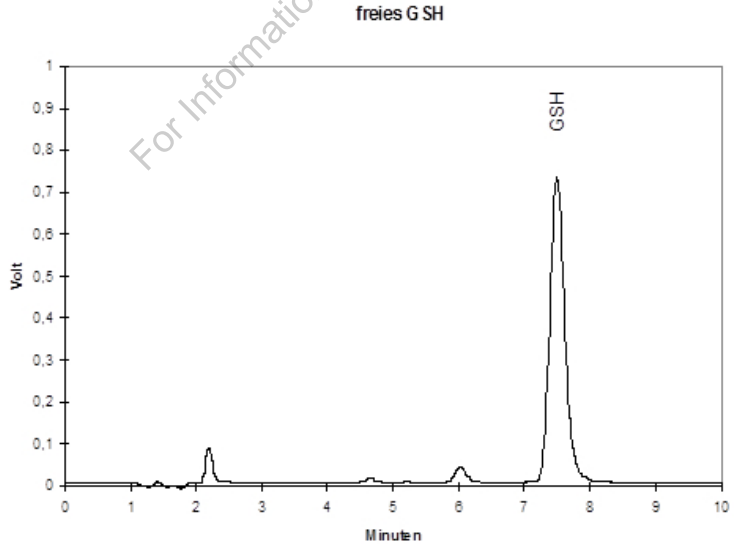
$$\text{Sample concentration (nmol/l)} = \frac{\text{Peak height sample} \times \text{calibrator concentration*}}{\text{Peak height calibrator}}$$

* see specification data sheet

Tip: Alternatively, the peak area instead of the peak height can be used for quantification.

Typical chromatograms





12. LIMITATIONS

Do not use serum or plasma samples. The content of glutathione in serum and plasma is lower, and it is not possible to distinguish between the oxidised and reduced glutathione form. Do not use lipaemic samples.

13. QUALITY CONTROL

Please keep in mind that all data mentioned here are referring to reduced glutathione (GSH or total GSH).

Reference range

We recommend each laboratory to establish its own reference range.

Controls

Control samples should be analysed with each run. Results, generated from the analysis of control samples, should be evaluated for acceptability using appropriate statistical methods. The results for the samples may not be valid if within the same assay one or more values of the quality control sample are outside the acceptable limits.

14. PERFORMANCE CHARACTERISTICS

Please keep in mind that all data mentioned here are referring to reduced glutathione (GSH or total GSH).

Precision and reproducibility

Intra-Assay (n = 12)

- Total GSH: 3.9 % (577 µmol/l)
- Reduced glutathione: 3.3 % (360 µmol/l)

Inter-Assay (n = 12)

- Total GSH: 4.2 % (544 µmol/l)
- Reduced glutathione: 3.3 % (108 µmol/l)

15. DISPOSAL

The mobile phase (MOPHA), reduction solution (REDSOL), internal standard (INT STD), and derivatisation solution (DER) must be disposed as non-halogenated solvents. The precipitation reagent (PREC) can be neutralised with NaOH to pH 7.0 and disposed as salt solution.

Important: Reaction will produce heat, be careful!

Please refer to the appropriate national guidelines.

16. TROUBLESHOOTING

Problem	Possible cause	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 imprecise	Check pump, degas the system
	System is not in steady state yet	Rinse system mobile phase for 15 min

Problem	Possible cause	Solution
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 imprecise	Check pump, degas the system
Baseline is not smooth	Pump delivers imprecise	Check pump, degas the system
	Detector flow cell is dirty	Clean flow cell

17. REFERENCES

1. Chawla, R. et al., 1984. Plasma cysteine, cystine, and glutathione in cirrhosis. *Gastroenterology*, **87**(4), pp.770–776.
2. Henning, S.M. et al., 1991. glutathione blood levels and other oxidant defense indices in men fed diets low in vitamin C. *The Journal of nutrition*, **121**(12), pp.1969–1975.
3. Meister, A., 1995. Mitochondrial changes associated with glutathione deficiency. *Biochimica et biophysica acta*, **1271**, pp.35–42.

18. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- The test components contain organic solvents. Contact with skin or mucous membranes must be avoided.
- All reagents in the kit package are for research use only.
- Reagents should not be used beyond the expiration date stated on kit label.
- Do not interchange different lot numbers of any kit component within the same assay.
- The guidelines for laboratories should be followed.
- Incubation time, incubation temperature and pipetting volumes of the components are defined by the producer. Any variation of the test procedure,

which is not coordinated with the producer, may influence the results of the test. Immundiagnostik AG can therefore not be held responsible for any damage resulting from incorrect use.

- Warranty claims and complaints regarding deficiencies must be logged within 14 days after receipt of the product. The product should be send to Immundiagnostik AG along with a written complaint.

Used symbols:


Temperature limitation

Catalogue Number

For research use only

To be used with


Manufacturer

Contains sufficient for <n> tests

Lot number


Use by


Attention


Consult instructions for use